THE AVIAN EGGSHELL : A MEDIATING BOUNDARY

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CONTENTS

INTRODUCTION

At the outset of the work discussed in this thesis, it was easy to catalogue many attributes of the avian eggshell - a barrier to microbial infection of the albumen, mechanical protection of the embryo, a reservoir of calcium for embryo bone formation, camouflage, porosity permitting the diffusion of respiratory gases, the conservation of water, etc. - but impossible to do other than speculate on the possible nature and extent of the interactions, if any, of these attributes. Indeed the paucity of information about the fine structure and mineralogy of eggshell was one impediment to an understanding of its overall contribution to the well-being of the embryo. The advent of scanning electron microscopy provided an opportunity for a thorough study of eggshell structure and the observations presented in Section 2 can be considered to be the major contribution so far in this area of avian biology. A classification of the pore systems in avian eggshells can be regarded as the focal point of these studies.

The classification of the pore systems was timely in that it complemented the classic studies of Professor Herman Rahn and his colleagues who calculated the effective pore area from determinations of the water conductance of egg-shells. It is noteworthy that the allometric relationship of porosity and egg mass, for example, which Rahn noted does not reflect the pore systems outlined in Section 2. Attempts to account for this situation led (Section 3) to the proposition that the avenues in the shell along which respiratory gases diffuse ought to be considered as diffusion pathways and that each component of an egg's outer covering - the limiting membrane, two shell membranes, calcitic shell with its outer boundary of cuticle/cover - should be considered as a resistance in series with many others. As some of these resistances, especially the cuticle or cover, do not have an appreciable affect on the rate of diffusion of respiratory gases or water vapour across the shell, the

question arises : what contribution, if any, do they make to the well-being of the developing embryo? It is evident from the material presented in Section 3 that in some instances the cuticle/cover can be regarded as adaptations that protect the pores in the shell - the critical resistance from the viewpoint of the rate of diffusion of oxygen to the embryo - from occlusion by nest debris and preening oils. The studies on the water-proofing of the hens' eggshell revealed also that the cuticle plays an important role in the defence against microbial colonization of the yolk and white. As the evidence accumulates, it becomes more and more apparent that the avian eggshell is not merely a porous, mineralised container in which embryogenesis proceeds providing the brooding parent is an adequate source of heat. Indeed the current view is that the shell acts as a mediating boundary such that the embryo can "communicate" with the nest atmosphere but at the same time is protected from inimical features of the nest environment. Detailed study of the latter has been neglected but initial attempts to monitor it continously or study it retrospectively are discussed in Section 4.

The defence of the embryo against microbial infection is yet another neglected topic in avian biology. Although the evidence supports the concept of the shell as a mediating boundary, it is evident from the observations presented in Section 3 that it is not an infallible barrier to microbial infection of the egg contents. This directed attention to the antimicrobial properties of the albumen (Section 5). It is now evident that the chelation of iron by ovotransferrin is the principal means whereby contaminants of the albumen are prevented from growing and the yolk is thereby protected from decay.

SECTION 1

A LIST OF PUBLICATIONS

This section lists all the publications to which R.G. Board has contributed. The academic relationships of contributors to multi-authored papers are given in the appendix to this section.

1. AVIAN EGGS

(a) Shell structure & composition

1. Board, R.G. (1974a) Microstructure, water resistance and water repellency of the pigeon eggshell. Bri. Poult. Sci. 15, 415-419. (P)* [21]†

2. Board, R.G. (1974b) The microstructure of the cuticle-less shells of the eggs of the domestic hen. Bri. Poult. Sci. 16, 89-91. (P)

3. Board, R.G. (1981a) A non-destructive method for studying fine structure of the surface of eggshells. Bri. Poult. Sci. 22, 407-408. (P) [28]

4. Board, R.G. & Love, G. (1980) Magnesium distribution in avian eggshells. Comp. Biochem. Physiol. 66A, 667-672. (P) [10]

5. Board, R.G. & Love, G. (1983) Magnesium distribution in avian eggshells with particular reference to those of wildfowl (Anatidae). Comp. Biochem. Physiol. 75A, 111-116. (P) [11]

6. Board, R.G. & Perrott, H.R. (1979a) The plugged pores of tinamou (Tinamidae) and Jaçana (Jaçanidae) eggshells. Ibis, 121, 469-474. (P) [5]

7. Board, R.G. & Perrott, H.R. (1979b) Vaterite, a constituent of the eggshells of the non-parasitic cuckoos, Guira guira and Crotophagi ani. Calc. Tiss. Int. 29, 63-69. (P) [6]

8. Board, R.G., Perrott, H.R., Love, G. & Scott, V.D. (1983) The phosphate rich cover of the eggshells of Grebes. Submitted to J. Zool., London. (P) [7]

9. Board, R.G., Perrott, H.R., Love, G. & Seymour, R.S. (1982) A novel pore system in the eggshells of the Mallee fowl, Leipoa ocellata. J. exp. Zool. 220, 131-136. (P) [8]

* P = Research Papers RN = Research Notes R = Reviews
BC = Book Chapters EB = Edited Books B = Books
† = The order of the papers in the thesis

10. Board, R.G. & Scott, V.D. (1980) Porosity of avian eggshells. (R) [13]
Amer. Zool. 20, 339-349.

11. Board, R.G. & Tullett, S.G. (1975) The pore arrangement in (P) [3]
the emu (Dromaius novaehollandiae) eggshell as shown
by plastic models. J. Microscopy, 103, 281-284.

12. Board, R.G., Tullett, S.G. & Perrott, H.R. (1977) An arbitrary (P) [1]
classification of the pore system in avian eggshells.
J. Zool., Lond. 182, 251-265.

13. Perrott, H.R., Scott, V.D. & Board, R.G. (1981). The (P) [12]
orientation of calcite crystals in hen's eggshell.
Calcif. Tiss. Int. 33, 119-124.

14. Tullett, S.G. & Board, R.G. (1977) Determinants of eggshell (P) [9]
porosity. J. Zool., Lond. 183, 203-211.

15. Tullett, S.G., Board, R.G., Love, G., Perrott, H.R. & (P) [4]
Scott, V.D. (1976) Vaterite deposition during eggshell
formation in the cormorant, gannett and shag and in
the "shell less" eggs of the domestic fowl.
Acta Zool. (Stockhm.) 79, 79-87.

16. Tullett, S.G., Lutz, P.L. & Board, R.G. (1974) The fine (P) [2]
structure of the pores in the shell of the hen's egg.
Brit. Poult. Sci. 16, 93-95.

(b) Properties & functions of the shell & shell membranes

17. Board, R.G. (1980) The avian eggshell - a resistance network. (R) [31]
J. appl. Bact. 48, 303-313.

18. Board, R.G. (1981) The microstructure of avian eggshells, (P) [15]
adaptive significance and practical implications in
aviculture. Wildfowl, 32, 132-136.

19. Board, R.G. (1982) Properties of avian eggshells and their (R) [14]
adaptive value. Biol. Revs. 57, 1-28.

20. Board, R.G. & Halls, N.A. (1973a) The cuticle; a barrier to (P) [22]
liquid and particle penetration of the shell of the
hen's egg. Brit. Poult. Sci. 14, 69-97.

21. Board, R.G. & Halls, N.A. (1973b) Water uptake by eggs of (P) [23]
Mallards and Guinea Fowl. Brit. Poult. Sci. 14, 311-314.

22. Bond, G.M., Scott, V.D., Cooke, R.G. & Board, R.G. (1980) (P) [16]
Correlation of hatching techniques in some avian species
with the mechanical properties of their eggs.
SEB Symposium 34, 459-461.

23. Hoyt, D.F., Board, R.G., Rahn, H. & Paganelli, C.V. (1979) (P) [17]
The eggs of the anatidae: Conductance, pore structure
and metabolism. Physiol. Zool. 52, 438-450.

24. Sparks, N.H.C. & Board, R.G. (1983) Cuticle, shell porosity and water uptake by hens' eggshells. Submitted to Brit. Poult Sci. (P) [24]

25. Tranter, H.S., Sparks, N.H.C. & Board, R.G. (1983). A note on the structure and iron binding properties of eggshell membranes. Brit. Poult. Sci. 24, 123-130. (P) [42]

26. Tranter, H.S., Sparks, N.H.C. & Board, R.G. (1983) Changes in the limiting membrane and oxygen permeability of chicken eggshells during incubation. Brit. Poult. Sci. (In press). (P) [20]

27. Tullett, S.G. & Board, R.G. (1976) Oxygen flux across the integument of the avian egg during incubation. Br. Poult. Sci. 17, 441-450. (P) [19]

(c) Nest Environment

28. Board, R.G. & Perrott, H.R. (1982) The fine structure of the incubated eggshells of the Helmeted guinea fowl (Numidia meleagris) J. Zool., Lond. 196, 445-451. (P) [29]

29. French, N.A. & Board, R.G. (1983). Water vapour conductance of wildfowl eggs & incubator humidity. Wildfowl. 34, 144-152. (P) [18]

30. Howey, P., Board, R.G., Davis, D. & Kear, Janet (1983) The microclimate of waterfowl nests. Ibis. (In press). (P) [27]

31. Howey, P.W., Board, R.G. & Kear, J. (1977) A pulse position modulated multi-channel radio telemetry system for the study of the avian nest microclimate. Biotelemetry, 4, 169-180. (P) [26]

(d) Microbiology

32. Ayres, J.C., Kraft, A.A., Board, R.G., Torrey, G.S. & Rizk, S.S. (1967) Sanitation practices in egg handling and breaking plants and the application of several disinfectants for sanitizing eggs. J. appl. Bact. 30, 106-116. (P)

33. Board, P.A. & Board, R.G. (1967) A method of studying bacterial penetration of the shell of the hen's egg. Lab. Pract. 16, 471-473 (P) [25]

34. Board, P.A. & Board, R.G. (1968) A diagnostic key for identifying organisms recovered from rotten eggs. Brit. Poult. Sci. 9, 111-120. (P) [34]

35. Board, R.G. (1964) The growth of Gram-negative bacteria in the hen's egg. J. appl. Bact. 27, 350-364. (P) [38]

36. Board, R.G. (1965a) Bacterial growth and penetration of the shell membranes of the hen's egg. J. appl. Bact. 28, 197-205. (P) [37]

37. Board, R.G. (1965b) The properties and classifications of the predominant bacteria occurring in rotten eggs. J. appl. Bact. 28, 437-453. (P) [32]

38. Board, R.G. (1968a) Review article: The course of microbial infection of the hen's egg. J. appl. Bact. 29, 319-341 (R) [43]

39. Board, R.G. (1968b) Microbiology of eggs: a review. In Egg Quality: A study of the Hen's Egg (Ed. T.C. Carter). Oliver & Boyds: Edinburgh. (R) [45]

40. Board, R.G. (1969) The microbiology of the hen's egg. Adv. Appl. Microbiology 11, 245-281. (R) [44]

41. Board, R.G. (1973) "The microbiology of eggs" a chapter in "Egg Science & Technology" (Eds. W.J. Stadelman & O.J. Cotterill) Avi: Westport. (BC)

42. Board, R.G. (1977) "The microbiology of eggs" a chapter in the 2nd ed. of "Egg Science & Technology" (Eds. W.J. Stadelman & O.J. Cotterill) Avi: Westport. (BC)

43. Board, R.G. & Ayres, J.C. (1965) The influence of temperature on bacterial infection of the hen's egg. Appl. Microbiol. 13, 358-364. (P) [39]

44. Board, R.G., Ayres, J.C., Kraft, A.A. & Forsythe, R.H. (1964) The microbiological contamination of eggshells and egg packing materials. Poult. Sci. 43, 584-595. (P) [52]

45. Board, R.G. & Fuller, R. (1974) Non-specific antimicrobial defences of the avian egg, embryo and neonate. Biol. Rev. 49, 15-49. (R) [30]

46. Board, R.G. & Halls, N.A. (1973c) The effect of iron on the growth of Escherichia coli in albumen taken from the hen's egg. Brit. Poult. Sci. 14, 359-371. (P) [49]

47. Board, R.G. & Hornsey, D.G. (1978) Serum and egg white proteins in Chemical Zoology - Birds. Academic Press, New York. (R) [46]

48. Board, P.A., Hendon, L.P. & Board, R.G. (1968) The influence of iron on the course of bacterial infection of the hen's egg. Brit. Poult. Sci. 9, 211-215. (P) [41]

49. Board, R.G., Loseby, S. & Miles, V.R. (1979) A note on microbial growth of the avian eggshell. Brit. Poult. Sci. 20, 413-420. (P) [36]

50. Board, R.G. & Perrott, H.R. (1979c) A note on actinomycetes on a museum specimen of eggshell. J. Appl. Bact. 47, 539-541. (RN) [35]

51. Board, R.G. & Tranter, H.S. (1982) "The microbiology of eggs" a chapter in the 3rd Edition of "Egg Science & Technology" (Eds. W.J. Stadelman & O.J. Cotterill) Avi: Westport. (BC)

52. Board, R.G. & Wilson, E.G.H. (1965a) Coagulation of pasteurized whole egg due to the growth of Bacillus cereus. Edinb. Sch. Agric. exp. Work. pp. 89. (RN)

53. Board, R.G. & Wilson, E.G.H. (1965b) Level of microbial contamination of eggs used for hatching. Edinb. Sch. Agric. exp. Work pp. 89-90. (RN)

54. Seviour, E.M. & Board, R.G. (1972) Bacterial growth in albumen taken from the eggs of domestic hens and water fowl. Brit. Poult. Sci. 13, 557-575. (P) [48]

55. Seviour, E.M. & Board, R.G. (1972) The behaviour of mixed bacterial infections in the shell membranes of the hen's egg. Brit. Poult. Sci. 13, 33-43. (P) [40]

56. Seviour, E.M., Sykes, F.R. & Board, R.G. (1972) A microbiological survey of the incubated eggs of chicken and water fowls. Brit. Poult. Sci. 13, 549-556. (P) [33]

57. Tranter, H.S. & Board, R.G. (1982) The inhibition of vegetative cell outgrowth and division from spores of Bacillus cereus T by hen egg albumen. J. appl. Bact. 52, 67-73. (P) [50]

58. Tranter, H.S. & Board, R.G. (1982) Review. The antimicrobial defence of avian eggs: biological perspective and chemical basis. J. appl. Bioch. 4, 295-338. (R) [47]

59. Tranter, H.S. & Board, R.G. (1983) Influence of pH and iron deprivation on bacteria in hen egg-white. J. appl. Bact. (In press). (P) [51]

2. FOOD MICROBIOLOGY AND OTHER TOPICS

60. Banks, J.G. & Board, R.G. (1982a) Comparison of methods for the determination of free and bound sulpher dioxide in stored British fresh sausage. F. Sci. Food Agric. 33, 197-203. (P)

61. Banks, J.G. & Board, R.G. (1982b) Sulfite-inhibition of Enterobacteriaceae including Salmonella in British fresh sausage and in culture systems. J. Food Protection 45, 1292-1297. (P)

62. Banks, J.G. & Board, R.G. (1983a) The incidence and level of contamination of British fresh sausages and ingredients with salmonellas. J. Hyg., Camb. 90, 213-223. (P)

63. Banks, J.G. & Board, R.G. (1983b) The classification of pseudomonads and other aerobic Gram-negative bacteria from fresh and processed meat. Systematic and Applied Microbiology (In press). (P)

64. Board, R.G. (1967) A method of demonstrating the morphology of caulobacters to students. Lab. Pract. 16, 476. (RN)

65. Board, R.G. (1983) A modern introduction to food microbiology. Blackwells: Oxford. (B)

66. Board, R.G. (1968) Biological aspects of organic waste treatment. Journal of the Institute of Water Pollution Control, 67, 614-621. (R)

67. Board, R.G., Egar, E.L., Young, L.Y. & Wilson, E.G.H. (1966) A microbiological survey of British fresh sausages. J. appl. Bact. 29, ii. (RN)

68. Board, R.G. & Holding, A.J. (1960) The utilization of glucose by aerobic Gram-negative bacteria. J. appl. Bact. 23, XI. (RN)

69. Dalton, H.K., Board, R.G. & Davenport, R.R. (1983). The yeasts of British fresh sausages. Submitted to Ant. von. Leeuw. (P)

70. Davidson, C.M., Dowdell, M.J. & Board, R.G. (1973) Properties of Gram-negative aerobes isolated from meats. J. Food Sci. 38, 303-305. (P)

71. Dowdell, M.J. & Board, R.G. (1967). Changes in microflora during storage of sausages. J. appl. Bact. 30, ii. (RN)

72. Dowdell, M.J. & Board, R.G. (1968) A microbiological survey of British fresh sausages. J. appl. Bact. 31, 378-396. (P)

73. Dowdell, M.J. & Board, R.G. (1969) The microbiology of sausages. BFMIRA Technical Bulletin No. 440. (P)

74. Dowdell, M.J. & Board, R.G. (1971) The microbial association of British fresh sausage. J. appl. Bact. 34, 317-337. (P)

75. Halls, N.A. & Board, R.G. (1973) The microbial associations developing on experimental filters irrigated with domestic sewage. J. appl. Bact. 36, 465-474. (P)

76. Lennard, M., Robb, J. & Board, R.G. (1963) Fishy odour in milk. Experimental work., School of Agriculture, Edinburgh pp 67 (RN)

77. Board, R.G. & Carr, J.G. (1976) A guide to contributors. J. appl. Bact. 40, 1-22 (R)

78. Board, R.G. (1982) A guide for contributors. J. appl. Bact. 52, Supplement. (R)

79. 1971 The Isolation of Anaerobes. S.A.B. Technical No. 5. Ed. D.A. Shapton and R.G. Board. Academic Press: London. (EB)

80. 1972 Safety in Microbiology. S.A.B. Technical Series No. 6. Ed. D.A. Shapton and R.G. Board. Academic Press: London. (EB)

81. 1973 Sampling: The Microbiological Monitoring of Environments. S.A.B. Technical Series No. 7. Ed. R.G. Board and D.M. Lovelock. (EB)

82. 1974 Methods in Microbiological Assay. S.A.B. Technical Series No. 8. Ed. R.G. Board and D.M. Lovelock. (EB)

83. 1980 Microbiological Classification and Identification. Ed. M. Goodfellow and R.G. Board. Academic Press: London (EB)

3. SUMMARY

Research papers	54	Books	1
Reviews	12	Book chapters	3
Research notes	8	Edited books	5
TOTALS	74		9

APPENDIX TO SECTION 1

ACADEMIC RELATIONSHIPS OF CONTRIBUTORS TO MULTI-AUTHORED PAPERS

Colleagues

Professor J.C. Ayres, Dr. J.G. Carr, Dr. R.G. Cooke, Dr. R.R. Davenport, Mr. D. Davis, Dr. R. Fuller, Dr. R.H. Forsythe, Dr. M. Goodfellow, Professor A.J. Holding, Mr. D.G. Hornsey, Dr. D.F. Hoyt, Dr. Janet Kear, Dr. A.A. Kraft, Mrs. M. Leonard, Dr. G. Love, Mr. D.H. Lovelock, Dr. P.L. Lutz, Dr. C.V. Paganelli, Mr. H.R. Perrott, Professor H. Rahn, Miss J. Robb, Dr. V.D. Scott, Dr. R.S. Seymour, Mr. D.A. Shapton & Miss E.G.H. Wilson.

Research Assistants

Mrs. P.A. Board, Mrs. M.J. Dowdell, Miss E.L. Egar, Dr. N.A. Halls, Dr. P. Howey, Mrs. E.M. Seviour, Mrs. F.L. Sykes & Dr. L.Y. Young.

Research Students

Mr. J.G. Banks, Miss H.K. Dalton, Mr. N.A. French, Mr. L.P. Hendon, Mr. S. Loseby, Mr. U.R. Miles, Dr. S.S. Rizk, Mr. N.H.C. Sparks, Dr. H.S. Tranter & Dr. S.G. Tullett.

SECTION 2

SHELL STRUCTURE AND COMPOSITION

Although the structure of avian eggshells has been studied intermittently for more than a century (e.g. von Nathusius*) progress was hindered by the technical limitations of the light microscope. Electron optics are ideally suited to studies of mineralised structures such as eggshells, especially if examination of fine structure is combined with electron probe analysis and X-ray diffraction studies. This section contains off-prints that have contributed to the first ever classification of pore systems in avian eggshells (viz. Board et al., 1977; Board & Scott, 1980). Moreover the X-ray diffraction studies have shown, again for the first time, that substances other than calcite contribute to certain parts of avian eggshells. Thus, for example, the biologically unusual form of calcium carbonate, Vaterite, is a principal component of the surface layer of the shells of two species of non-parasitic cuckoos (Board & Perrott, 1979b).

In other eggshells - for example, those of the megapodes (Board et al., 1982) - calcium phosphate, perhaps in the amorphous form, is the principal mineral in the outer layer. Two fundamental questions are posed by these observations. What are the control mechanisms in the oviduct that determine shell porosity and mineral composition? Do the various pore systems of the proposed classification have any adaptive value? As the processes leading to calcite formation in the shell glands of birds are poorly understood, there is little prospect at the moment of any progress in our understanding of the factors which are associated with a switch from the deposition of one mineral form to another.

Nevertheless an attempt has been made (Tullett & Board, 1977) to define control mechanisms that influence shell porosity. The question of the adaptive value, if any, of the various pore forms seen in avian eggshells is considered in Sections 3, 4 and 5.

* The collected works of Wilhelm von Nathusius on avian eggshells have been translated by C. Tyler (1964; The University: Reading).

Reprinted from *J. Zool., Lond.* (1977) **182,** 251–265

An arbitrary classification of the pore systems in avian eggshells

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(*Accepted 17 August 1976*)

(With 4 plates and 1 figure in the text)

An arbitrary classification of the pore systems in shells has been based on the observations made during a survey with a scanning electron microscope of the eggs of *c.* 60 avian species. The classification is intended primarily to aid comparative studies of shell function and field studies of the overall contribution of the shell to the embryo's well-being. The location and form of the external orifice of the pore was used to define four major types of pore systems: (1) outer orifice open—the pore canal terminates at the surface of the shell; (2) outer orifice occluded—the pore canal terminates at the surface of the shell but the orifice is occluded by amorphous, waxy material; (3) outer orifice capped—the pore canal terminates at the outer edge of the true shell and its orifice is capped and, in many species, plugged by a stratum of vesicles, either organic or inorganic, which clothes the entire surface of the shell, and (4) outer orifice reticulate—the pore canal terminates at the edge of the column layer and its orifice is covered by a calcareous stratum containing a plexus of tubules. Each group was further divided on the form of the pore canals; shells having unbranched pore canals were put into one category and those in which some pores branched into another.

Contents

Introduction

Although studies of structure have provided much information about the morphology of pores in avian eggshells (von Nathusius (see Tyler, 1964*a*); Schmidt, 1962*a*, *b*; Tyler, 1969*b*; Erben, 1970; Simons, 1971), those concerned with function have tended to limit discussion to just one type (Freeman & Vince, 1974). The stylized pore of the text-books

TABLE I

The species of birds included in this study and the classification of their eggshells

		Source of eggs*
1. *Outer orifice open*		
(a) Pore canals unbranched:		
Pigeon: Fantail, Racing	(*Columba* spp.)	(2)
Wood	(*Columba palumbus*)	(3)
Dove: Cape		
Collared	(*Streptopelia decaocto*)	(3)
Diamond	(*Geopelia cuneata*)	(2)
(b) Pore canals branched and unbranched:		
Ostrich	(*Struthio camelus*)	(6)
2. *Outer orifice occluded*		
(a) Pore canals unbranched:		
Blackbird	(*Turdus merula*)	(3)
Bluetit	(*Parus caeruleus*)	(3)
Budgerigar	(*Melopsittacus undulatus*)	(2)
Common gull	(*Larus canus*)	(13)
Derbian parakeet	(*Psittacula derbyana*)	(9)
Giant petrel	(*Macronectes giganteus*)	(10)
Herring gull	(*Larus argentatus*)	(13)
House sparrow	(*Passer domesticus*)	(3)
Kestrel	(*Falco tinnunculus*)	(1)
Lanner falcon	(*Falco biarmicus*)	(6)
Linnet	(*Acanthis cannabina*)	(3)
Manx shearwater	(*Puffinus puffinus*)	(13)
Owl: Little	(*Athene noctua*)	(2)
Tawny	(*Strix aluco*)	(2)
Oystercatcher	(*Haematopus ostralegus*)	(13)
Peregrine falcon	(*Falco peregrinus*)	(1)
Pukeko	(*Porphyrio melanotus*)	(17)
Razorbill	(*Alca torda*)	(13)
Red-legged partridge	(*Alectoris rufa*)	(5)
Ring-necked pheasant	(*Phasianus colchicus*)	(9)
Robin	(*Erithacus rubecula*)	(3)
3. (*continued*)		
Bobwhite quail	(*Colinus virginianus*)	(9)
Cereopsis goose**	(*Cereopsis novaehollandiae*)	(5)
Domestic fowl	(*Gallus domesticus*)	(8)
Flamingo: Andean	(*Phoenicoparrus andinus*)	(5)
Chilean	(*Phoenicopterus chilensis*)	(5)
Greater	(*Phoenicopterus roseus*)	(5)
Rosy	(*Phoenicopterus ruber*)	(5)
Grey heron	(*Ardea cinerea*)	(10)
Guinea fowl	(*Numida meleagris*)	(8)
Japanese quail	(*Coturnix coturnix*)	(12)
Moorhen	(*Gallinula chloropus*)	(5)
Peahen	(*Pavo cristatus*)	(2)
Penguin: Black-footed	(*Spheniscus demersus*)	(6)
Gentoo	(*Pygoscelis papua*)	(11)
King**	(*Aptenodytes patagonica*)	(1, 11)
Sarus crane	(*Grus antigone*)	(6)
Turkey	(*Meleagris gallopavo*)	(7)
**Tendency for lanceolate pores but not usually branched.		
	(ii) Inorganic capping material:	
Cormorant	(*Phalacrocorax carbo*)	(6)
Gannet	(*Sula bassana*)	(10, 14)
Shag	(*Phalacrocorax aristotelis*)	(13)
(b) Pore canals branched and unbranched	(i) organic capping material:	
Emperor penguin	(*Aptenodytes forsteri*)	(6, 10)
Tendency for lanceolate pores and others showing distinct branching		
	(ii) Inorganic capping material:	
No eggs of this type included in this study		
4. *Outer orifice reticulate*		
(a) Pore canals unbranched:		
Buzzard	(*Buteo buteo*)	(1)
Osprey	(*Pandion haliaetus*)	(1)

2. (*continued*)

Sacred ibis	(*Threskiornis aethiopicus*)	(6)
Secretary bird	(*Sagittarius serpentarius*)	(1)
Snow petrel	(*Padrogoma nivea*)	(10)
Starling	(*Sternus vulgaris*)	(3)

(b) Pore canals branched and unbranched:

Greater rhea	(*Rhea americana*)	(6)

3. *Outer orifice capped*

(a) Pore canals unbranched (i) organic capping material:

Aylesbury duck	(*Anas platyrhynchos*)	(2)
Bantam hen	(*Gallus domesticus*)	(2)
Black swan	(*Cygnus atratus*)	(5)

4. (*continued*)

Sparrowhawk	(*Accipiter nisus*)	(1)
Stork: Open-billed	(*Anastomus oscitans*)	(15)
White	(*Ciconia alba*)	(6)
Yellow-billed	(*Mycteria ibis*)	(15)

(b) Pore canals branched and unbranched:

Cassowary	(*Casuarius casuarius*)	(16)
Emu	(*Dromaius novaehollandiae*)	(6)

5. *Unclassified*

Moa	Pores branched and unbranched	(17)
Aepyornis		(18)

* Source of eggs

(1) British Museum (Mr C. J. O. Harrison)
(2) Local fanciers
(3) Deserted nests/predation
(4) Game Conservancy, Fordingbridge (Dr G. V. Beer)
(5) Wildfowl Trust, Slimbridge (Prof. G. V. T. Matthews)
(6) London Zoo (Mr P. J. Olney)
(7) British United Turkeys (Dr R. K. Edson)
(8) Farms (Mr & Mrs S. H. Hoddinott)
(9) Bird Gardens (Mr M. Greene)
(10) Monks Wood Experimental Station (Drs A. S. Cooke, J. L. F. Parslow and P. J. Tilbrook)
(11) Edinburgh Zoo
(12) National Institute for Research in Dairying, Reading (Dr R. Fuller)
(13) Bardsey Island Bird Observatory (Mr M. Peacock)
(14) Bass Rock, Scotland (Sir Huw Dalrymple-Hamilton & Mr F. Marr)
(15) MRC, Kisumu, Kenya (Dr J. Parsons)
(16) Calderpark, Glasgow Zoo (Mr R. J. P. O'Grady)
(17) Christchurch Museum (N.Z.) (Mr R. J. Scarlett)
(18) South Africa (Mr G. S. Cubbitt)

has the geometry of a golf tee, the companulate orifice being outermost, and though this shape is common in eggshells (Tyler, 1964*b*, 1965, 1969*a*) it is not unique (Tyler, 1966; Tyler & Simkiss, 1959) nor lacking modifications which may be associated with shell function. As the function of a pore may be likened to that of stomata in leaves, it would seem reasonable to assume that pores have been modified so that the eggshell is fitted to the environment obtaining in the nest both before and during incubation. Such a concept has been discussed elsewhere (Board & Halls, 1973*a*, *b*; Board, 1974, 1975; Tullett, Lutz & Board, 1975); Board & Fuller, 1974; Tullett, Board *et al.*, 1976). Thus the shell at oviposition has, as a result of its complement of pores, a diffusion capacity (Kutchai & Steen, 1971; Tullett & Board, 1976) which matches one of the principal demands of the embryo, an adequate supply of O_2 (Wangensteen, Wilson & Rahn, 1971; Wangensteen & Rahn, 1971). It is probable that modifications of the pore system in response to environmental pressures has maintained the diffusion capacity but minimized the risk of the embryo being crushed, desiccated, asphyxiated, infected or infested (Board & Fuller, 1974).

Although the germ of the hypothesis has been considered by those interested primarily in the overall biology of birds viz., Lack (1968) in the chapter "Problems concerned with eggs", such discussions appear to have been hampered by a lack of appreciation of the fine structure of the eggshell. Although there is much information on the fine structure as seen with the light microscope (Tyler, 1969*b*), this has tended to be used in discussions of phylogeny and rarely, if ever, in a consideration of the overall function of the shell. In the present study, electron optics were used to survey eggshell structure with the objective of establishing an arbitrary classification which would encourage comparative and field studies of shell function.

Materials

The source of the eggs is given in Table I.

Methods

Pieces of untreated shell or shell which had been scoured with boiling NaOH (5% w/v) were cemented (DAG 915; Acheson Colloid Company, Prince Rock, Plymouth) to aluminium stubs and coated under vacuum with gold-palladium. A scanning electron microscope (SEM; Stereoscan S4; Cambridge Scientific Instruments Ltd.) with an accelerating voltage of 10 kV was used to study the shells.

Plastic models

Plastic models of coarse shells were prepared by the method of Board & Tullett (1975) a modification of that of Tyler (1956). As the repeated drawing and releasing of a vacuum destroyed the structure of fine shells, another method was devised in which Plasticraft (Turner Research Ltd., Leeds) was mixed thoroughly with the catalyst, entrained gas bubbles were removed under vacuum and the plastic diluted by a third with amyl alcohol. The diluted plastic was poured slowly into polythene specimens tubes containing pieces of shell soaked in amyl alcohol. The alcohol was evaporated and the plastic polymerized by storage at 50°C for 18–24 hours. The shell was decalcified with concentrated HCl or ethylene-diaminetetra acetic acid (5 or 10% w/v, neutralized with NaOH). For examination with the SEM, plastic models were coated with carbon and then with gold-palladium.

Results

In all the eggshells included in the study (Table I), the pores originated in the valleys of the cone layer. The factors determining their numbers and distribution have been discussed elsewhere (Tullett, 1975). The location and form of the external orifice of the pore canal was used to define four main types of pore systems (Fig. 1). Within each type

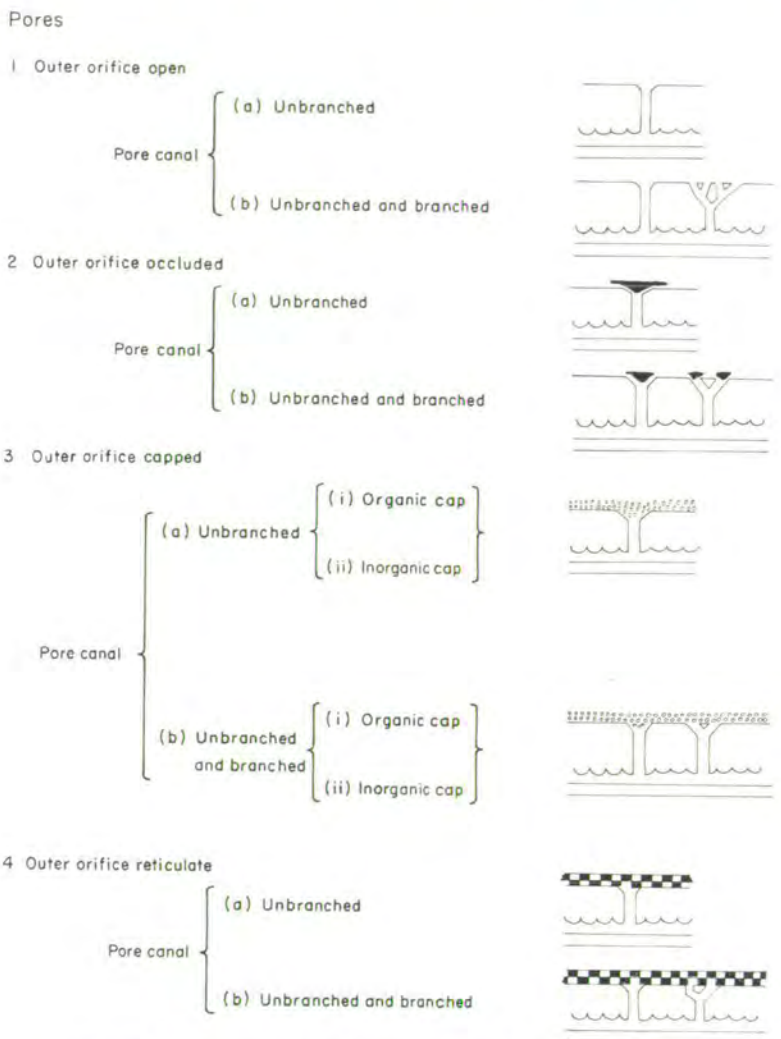

FIG. 1. A classification of the pore systems in avian eggshells.

further division was based on features such as whether or not the pore canals branched and the chemical composition of the spheres capping the pores in Type 3 (Fig. 1). If the overall organization of the shell is considered, then two major types of shell can be recognized: (a) porous shells—the pore canal terminated at the outer edge of the column layer on whose surface there may or may not be a layer of amorphous material, and

(b) capillary-porous shells—the lumen of the pore vented externally into a stratum of spheres or a plexus of tubules. In the following discussion, the various types of pore systems are considered in one or other of these two groups. As with all classifications of biological systems, the available information tended to give a spectrum so it was necessary to select arbitrarily the criteria for the classification given in Fig. 1. This is probably of little concern since the objectives of this study did not include a classification which reflects phylogenetic relationships.

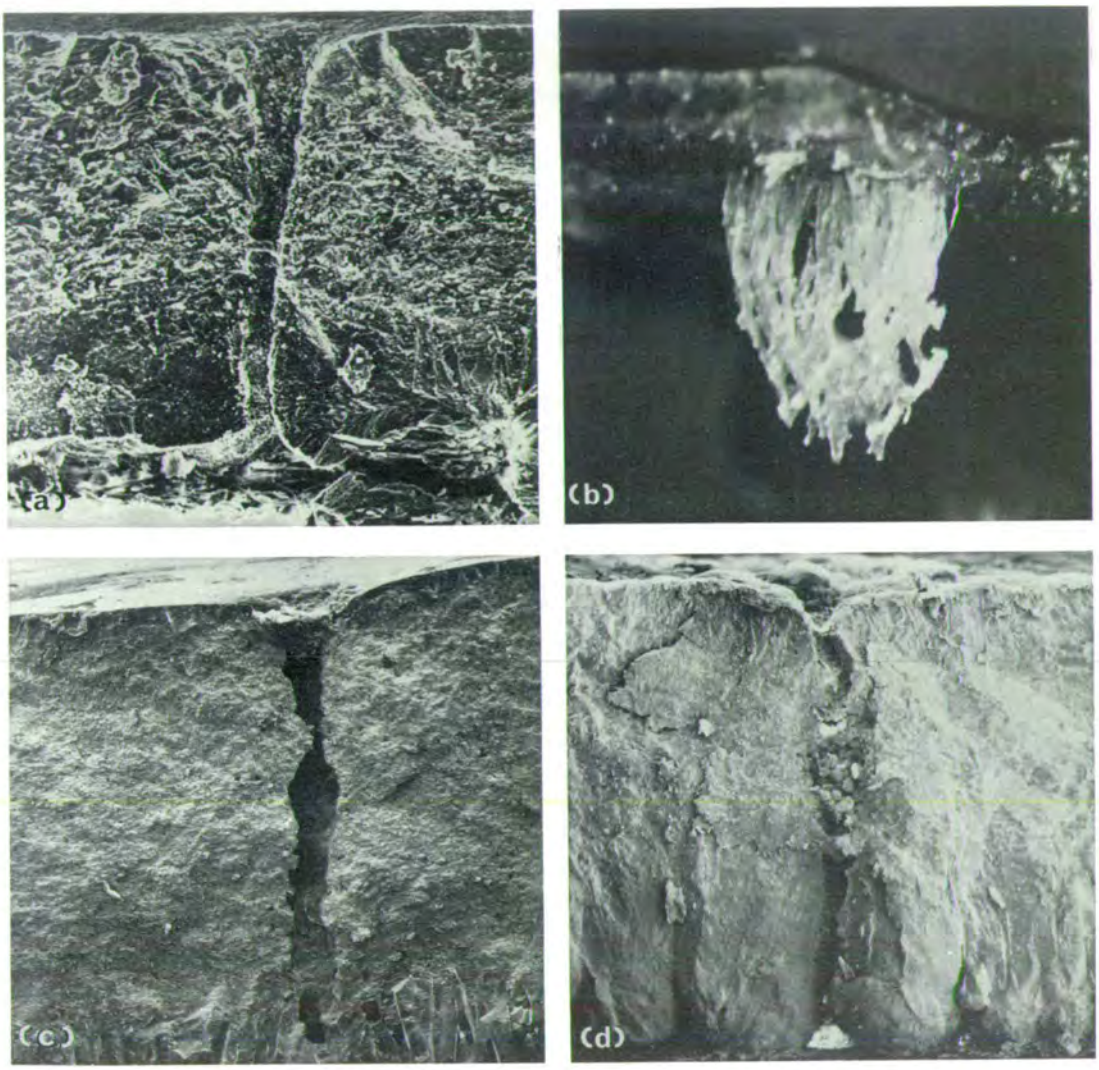

PLATE I. (a) Radially fractured pigeon eggshell showing outer pore orifice venting directly to environment (×435). (b) Plastic model showing extensive branching of pore system from the inner (bottom) to the outer (top) surface of an Ostrich eggshell (×18). (c) Radially fractured Rhea eggshell (×90). Subsidence of the surface crystal layer gives the impression of a pore plug. However, the pores are typically occluded. (d) Radially fractured Sarus crane eggshell showing occluded outer (top) pore orifice (×105).

Porous eggshells

In this type of eggshell, a pore originating in the cone layer traversed radially the column layer and its outer orifice was open or occluded with amorphous material.

Open pores

In the simplest pore form (Fig. 1) the external orifices of the unbranched pore canals were open. As noted by Board (1975), this was a characteristic feature of the eggshells of Racing pigeons, Fantail pigeons and the progeny of interbreeding between the two (Plate I(a)). In this survey, a similar arrangement was found in the eggshell of Wood pigeons (*Columba palumbus*) and Collared dove (*Streptopelia decaocto*). With birds nesting in dovecotes, brooding resulted in extraneous material being lodged in the pore canal but there was no evidence of the external orifice of the pores being occluded.

In addition to unbranched pores, the shell of the ostrich (*Struthio camelus*) contained a large number which branched extensively (Plate I(b)), a feature noted by von Nathusius (1868) and Tyler & Simkiss (1959) and by Sauer (1972) who examined the shells of extinct members of this family. Thus an opening in the valley of the cone layer was connected via a rhizoid-like arrangement of branched tubes to a saucer shaped depression in the external surface of the shell. The outer orifices of the pore canals were open (Plate IV(d)) in the eggshells we examined. The canals in the eggshells of the moa and *Aepyornis* branched less frequently than those of the ostrich and the branching tended to be limited to one plane only. Thus the outer orifices of the terminal branches were aggregated in groove(s) rather than saucer-shaped depressions in the shell's surface. It was noteworthy also that some pores fanned out but did not divide into two canals. Although the incidence of branched pores tends to be greater in eggs with thick shells (Tyler, 1964*b*) thickness itself is not of primary importance although it does obviously provide the opportunity for branching to occur. Thus the extent of branching of the pore canals in the thick (*c.* 3·8 mm) shell of *Aepyornis* egg was much less than in the thinner (*c.* 1·9 mm) ones of the ostrich. This observation, together with the one that branching is not confirmed to one plane, implies that branching is subject to control during the formation of the ostrich eggshell and is thus a feature favoured by natural selection.

The pore systems of the moa and *Aepyornis* eggshells have not been classified (Table I) because it could not be determined what modifications, if any, of the fine structure had occurred during the time (*c.* 2000 years with the *Aepyornis*) that the eggshells had been in the soil.

Occluded pores

The origin of the occluding material could not positively be identified. In no instance was any fine structure noted. It was unlikely therefore to be cuticle, which exhibits a vesicular structure, and was more probably material acquired during brooding. Eggshells in this category therefore may be mistaken for open pore types if examined as soon as they are laid. The contribution to the shiny appearance of the shell, its water repellency and tendency to become "charged" when examined with an SEM indicate that the occluding material contains waxes. In assigning eggshells to this category of the classification, particular emphasis was given to examination both of the surface of the shell and the external edge of radial sections with magnification up to 50 000 ×. The fine layer of occluding material was in marked contrast to the thick vesicular stratum in the capped

pore types (Plate I). There was great variation in the extent to which the outer orifice of the pore canal was occluded (Plate II). Thus with the oystercatcher (*Haematopus ostralegus*) there was a small amount only of material around the rims of the pore orifice; with the ring-necked pheasant (*Phasianus colchicus*) much of the pore opening was blocked, and

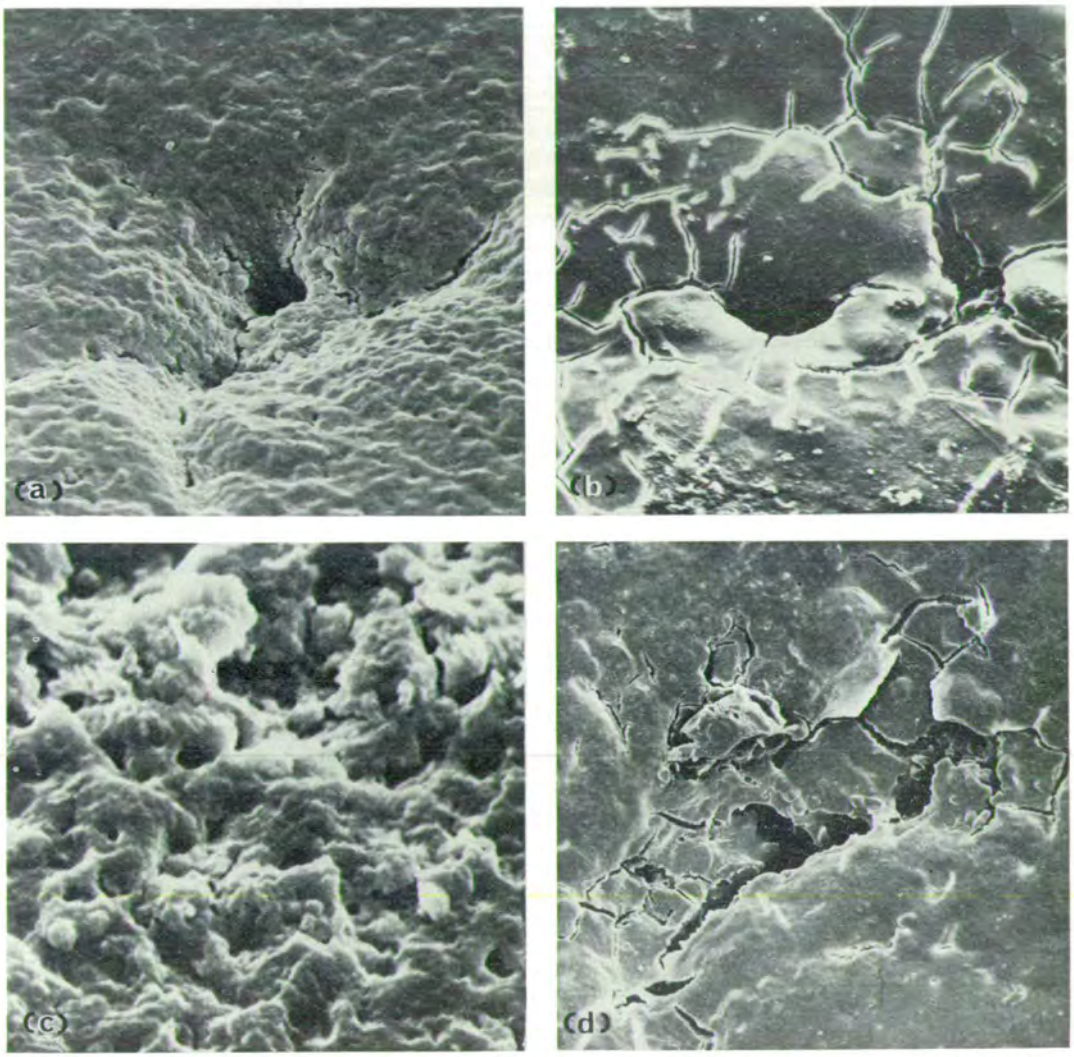

PLATE II. Outer surfaces of eggshells showing occluded pore orifices in (a) Oystercatcher (×1600), (b) Pheasant (×745), (c) Linnet (×5300), (d) Pheasant (×395).

with the blackbird (*Turdus merula*) the entire orifice was occluded. When the whole surface of the shell was covered with the occluding material, the latter contained fine fissures (e.g. the Blackbird), small holes (the Robin), or large cracks (the Common gull, *Larus canus*). As it is known that the transpiration characteristics of stomata are modified by waxes around or in the aperture (Martin & Juniper, 1970), eggshells containing pores

with much or little occluding material have been grouped together. Further sub-groups may well be defined when comparative studies of function, particularly the movement of water vapour, are done.

The eggshells of the Rhea provided the only example of the category (Fig. 1) in which the outer orifice was occluded and same pore canals were branched (Plate I(c)). The branching was limited; a pore canal either forked to give two tubes or merely fanned out into a lanceolate pore canal. The occluding material occurred as a plug set down in the pore orifice. It was noted that the branching of pores in the Moa and *Aepyornis* was in one plane. As fragments only of these shells were available it was not possible to establish the plane in relation to the whole egg. However, with the whole Rhea eggs the pores were clearly branched along the longitudinal axis.

Capillary-porous eggshells

Two distinct types of eggshell have been included in this major category. In one (the capped pores) the pore canal terminated at the outer surface of the column layer the surface of which was clothed with a stratum of spheres or, in the terminology of Simons (1971), vesicles. The change from the column layer to the stratum of spheres was abrupt and in SEM examination there was no difficulty in distinguishing between the capping material and the surface crystal layer of the true shell. With the other, the reticulate pores, the canal(s) originating in the cone layer did not traverse the entire shell. With the Emu (*Dromaius novaehollandiae*) (Board & Tullett, 1975) and Cassowary (*Casuarius casuarius*) (Plate III(c)), they terminated immediately below a layer of the shell which contained a network of tubules. These vented to the surface via many small pores. The shells of the eggs of three species of stork (Open-billed, *Anastomus oscitans*; Yellow-billed, *Mycteria ibis*; White stork, *Ciconia alba*) were composed of two distinct layers. The inner one was identified with the cone and column layer and this was traversed by pore canals. The outer layer, the actual thickness of which varied in the three species of stork, was composed of irregularly arranged crystalline material. This arrangement resulted in relatively large radial channels being connected tangentially by other channels thus forming a labyrinth. The lumen of the tubules was rich in dendritic crystals. Such crystals have not been seen in the tubules in the eggshells of Emu or Cassowary.

Tyler (1966) noted in studies with the light microscope that the pore canals in the eggs of some members (*Aquila* spp. eagles; the Accipitridae except *Gypaetus barbatus*, the Lammergeier and *Neophron percnopterus*, Egyptian vulture) of the Falconiformes terminated beneath the outer surface of the shell. We have (Plate III(d)) observed such pore arrangements in the eggshells of the Buzzard (*Buteo buteo*), Osprey (*Pandion haliaetus*) and Sparrowhawk (*Accipiter nisus*). No such system was found however, for example, in the kestrel (*Falco tinnunculus*), Peregrine falcon (*Falco peregrinus*) and Secretary bird (*Sagittarius serpentarius*).

The fine structure noted above warrants the recognition of this group of shells as capillary-porous bodies (Everett & Stone, 1958; Scheidegger, 1960; Luikov, 1966; Anon, 1972). Division of the two main shell types (the capped and reticulate pores) has been based on whether or not the pore canal forked (Fig. 1). With the capped pore, further division was based on the chemical composition of the capping material. Shells capped with spheres rich in vaterite (Tullett, Board *et al.*, 1976) were put into one category and those with mainly organic vesicles into another.

In eggshells having capped pores the capping material may merely bridge the outer orifice of the pore canal as in the Aylesbury duck, while in others it formed a plug (e.g. the Greater flamingo and in the Domestic fowl; see Tullett, Lutz & Board, 1975) which in the case of the guinea fowl extended far into the pore canal. Fissures were a common

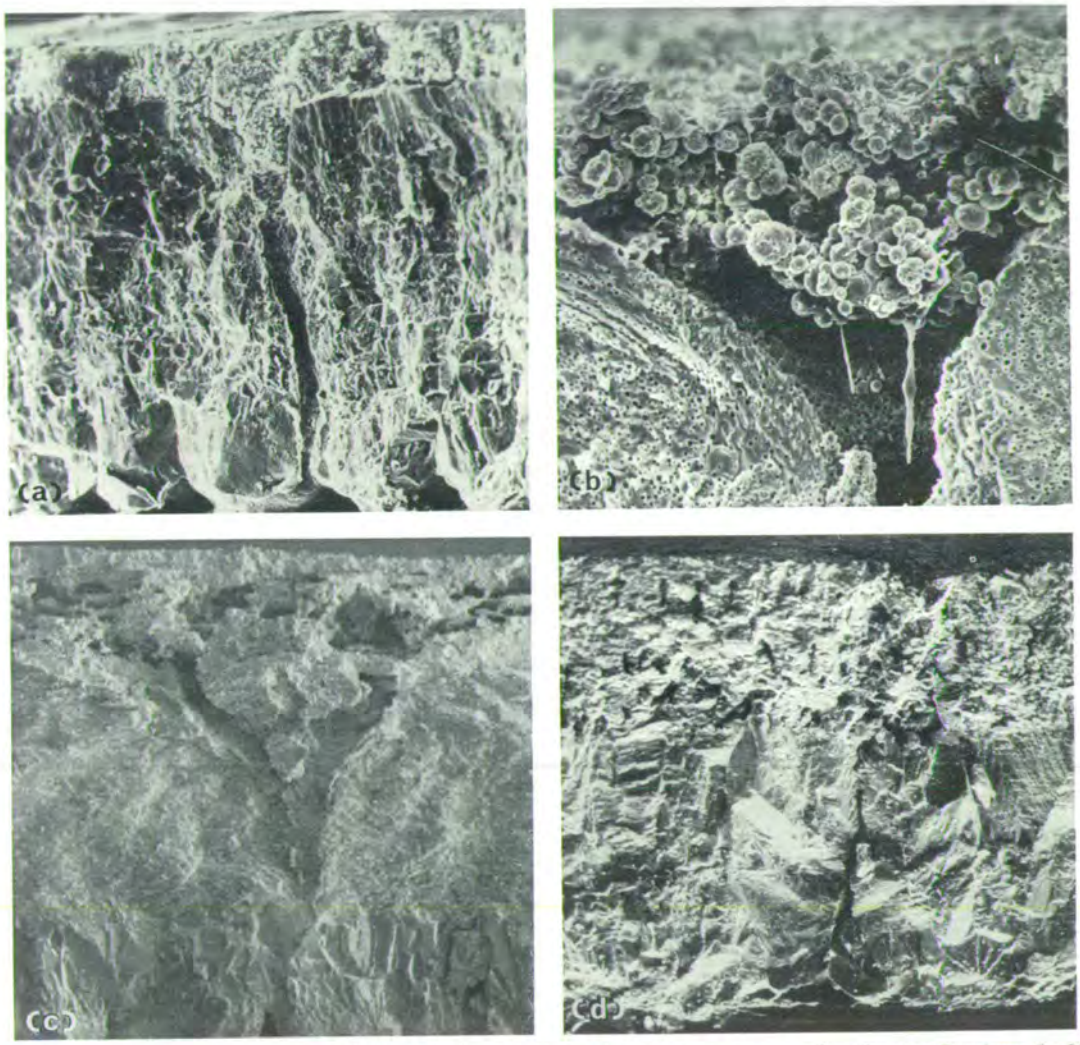

PLATE III. Radially fractured eggshells showing (a) Capped and plugged pore of a Greater flamingo (×90). (b) Cuticular plug in the outer pore orifice of a Grey heron (×880). (c) Reticulate shell of a Cassowary (×90). (d) Reticulate shell of an Osprey (×160).

feature of the surface of the unbrooded eggs of Domestic hens, Japanese quail (*Coturnix coturnix*), Guinea fowl (*Numida meleagris*) and flamingoes but not of the Gannet (*Sula bassana*), Shag (*Phalacrocorax aristotelis*) and Cormorant (*Phalacrocorax carbo*). In domesticated fowl, the fissures tended to be clustered and within a cluster there was a tendency for the individual fissures to radiate out from a common origin. Marshall &

Cruickshank (1938) referred to such arrangements as pore plaques. With quails and Guinea fowls, the fissures joined thus giving the impression of crazy-paving—Becking (1975) likened them to cracks in dried mud. Fissures were not evident in the surface of the shells of Guinea fowl eggs which had been brooded or polished with oil taken from the preening gland of Aylesbury ducks. It was noted, also, that the cover of the Guinea fowl egg absorbed readily grease from the fingers, the finger print being demonstrated easily with finger-print dust or immersion in water. In the latter case they stood out as brown markings on a whitish-grey background. These observations not only drew attention to a neglected component of the integument of the birds' egg but provide a warning

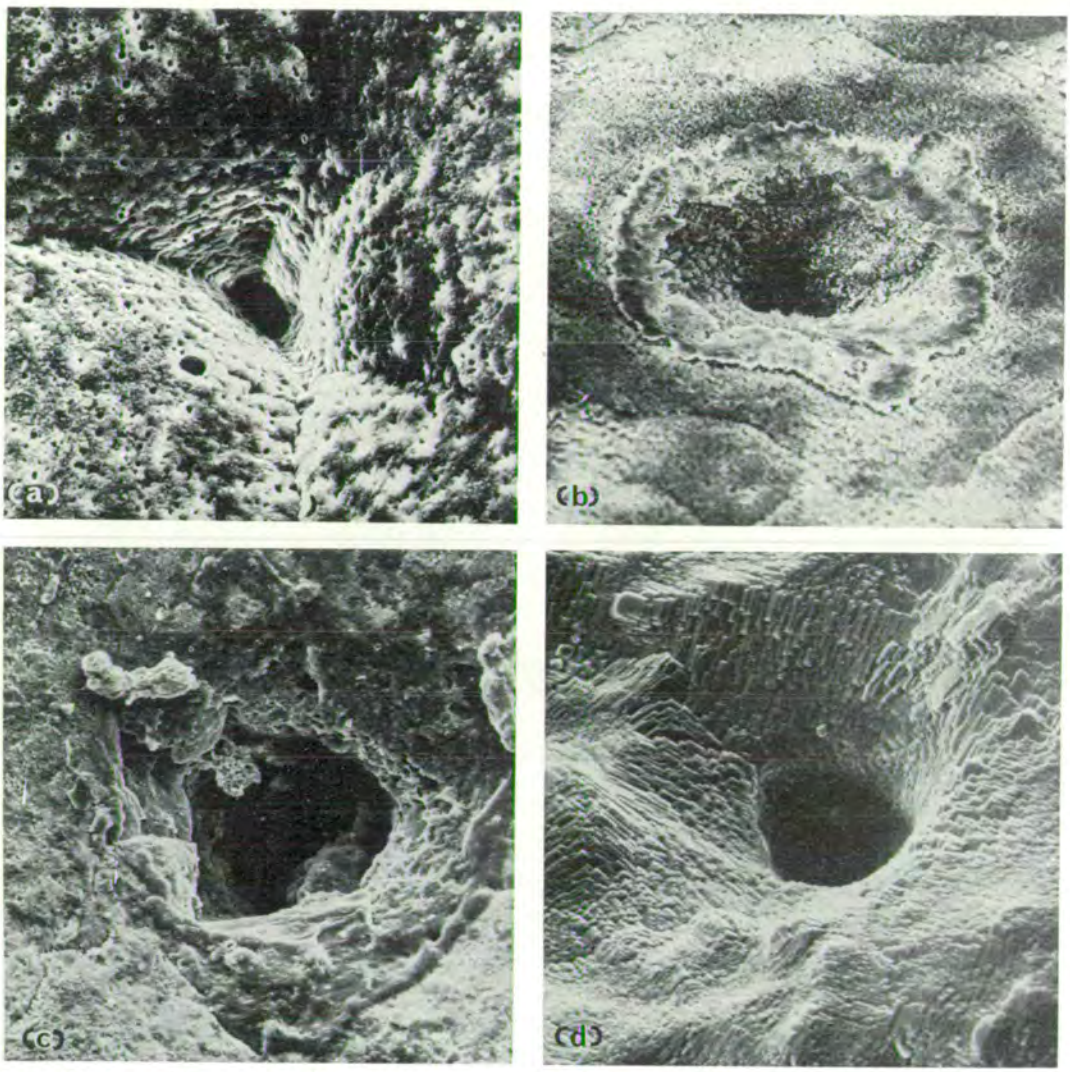

PLATE IV. Pore orifices "cleaned" by boiling in 5% (w/v) NaOH for 5 min. Viewed from the outer surface of (a) Blackbird eggshell (×785), (b) Starling eggshell (×385). Note ring of occluding material which has not been removed. (c) Naturally cuticle-less domestic fowl eggshell—not "cleaned" (×6680). (d) Ostrich eggshell (×1000).

against the uncritical interpretation of shell sections stained histochemically—if fat on the shell's surface is demonstrated, then was it there at oviposition, acquired during brooding or merely deposited by the fingers of those who handled the egg?

Geometry of pore orifice

The observations made in this study have been used to define relatively large categories of pore types. Within the categories other than the reticulate pore, and possibly the open pore, there was a marked diversity in the geometry of the outer pore orifice (Plate IV). As it is not justifiable to survey sufficient eggs of a wild species of bird to establish the extent of variation, the influence of selective pressure cannot be assessed. This variation is merely noted so that those who are concerned with the theoretical deduction of effective pore area are made aware of problems which may be encountered.

Discussion

If the results of this survey are considered along with those of Tyler (Tyler & Simkiss, 1959; Tyler, 1964*b*, 1965, 1966, 1969*a*), and Becking (1975), then we have information on the pore form in the eggs of more than 70 species of bird (Table II). Our classification may be considered to be tentative since it is based on such a small sample of the bird world (*c.* 8650 spp.). Nevertheless it should be of value to those who are concerned with comparative studies of shell function.

There has been a tendency for discussions of avian eggshells to be restricted arbitrarily to certain aspects. In consequence we have a paltry appreciation of the overall role of the shell, a mediating boundary between the bulk/nest environment and that obtaining within the egg. Thus discussions of structure and chemical composition (Schmidt, 1962*a*, *b*; Tyler, 1969*b*; Erben, 1970; Simons, 1971) have made little comment on the probable contributions which components make to the overall function of the shell, apart that is from recognizing the importance of the pores in the diffusion of O_2 and water vapour. In fact, there has been a tendency to take knowledge about the fine structure of the shell to augment that used to classify birds rather than to consider the concept that a shell plays an important role in fitting an egg to its environment. Those with a major interest in the biology of birds (Lack, 1968; Harrison, 1975) have appreciated this and attempted to account for gross morphological features of the egg in terms of camouflage, packing in nests, identification by parents, the "stability" of an egg on a rocky ledge, etc. Apart from noting that some eggs have chalky or shiny surfaces, their discussions have not encompassed an interpretation of the fine structure of the shell against a backcloth of the peculiarities of the bulk environment in which nesting occurs or the behaviour or life style of the parents. Studies of the physiology of the avian embryo have accorded such a cardinal role to the diffusion of O_2 and water vapour across the shell (Wagensteen, Wilson *et al.*, 1971; Ar *et al.*, 1974), that the defence of the embryo against asphyxiation, infection and infestation and its requirements for protection by a strong shell (Carter, 1971) have not been given even subordinate status (Board & Fuller, 1974).

If for the purposes of this discussion, this cardinal role of the pores is accepted, then it would seem reasonable to assume that the types considered in this communication have evolved so that eggshells are adapted to environments which, either before or during brooding, have inimical components which could operate via a naked pore to the detriment of the embryo. Although the nature of such components can be for the moment the

subject of speculation only, the concept is worthy of discussion because it may provide clues for those studying the egg in commerce or nature. Thus with the cuticle (Board & Halls, 1973*a*, *b*; Board, 1974, 1975), it has been suggested that through endowing the shell with water resistance, this structure fits an egg to an open nest where there is probably a

TABLE II

Classification of other shells based on the work of Becking (1975), W. von Nathusius (see Tyler, 1964a), Tyler (1965, 1966, 1969a) and Tyler & Simkiss (1959)

2(a) or 3(a) *Occluded or capped pores*	
Occluding material probably organic:	
Andean condor	*Vulture gryphus*
Ashy tailor bird	*Orthotomus sepium*
Black vulture	*Coragyps atratus*
Californian condor	*Gymnogyps californius*
Egyptian vulture	*Neophron percnopterus*
Falcons	*Falco* spp.
Galliformes	e.g. *Arborophila brunneopectus* *Perdix perdix*
Great reed warbler	*Acrocephalus arundinaceus*
Gruiformes	e.g. *Turnix suscicator*
Horsfield's Jungle babbler	*Trichastoma sepiaruim*
Kiwi	Apterygidae
Lammergeier	*Gypaetus barbatus*
Loons	Gaviiformes
New World vultures	Carthartidae
Parasitic cuckoos	Cuculidae
South American King vulture	*Sarcorhampus papa*
Tinamou	*Eudiana elegans* *Nothocercus bonapartei* *Tinamus salvini*
Tubenoses	Procellariiformes
Wildfowl (larger swans, e.g. Black-necked, Mute, Whistling and Whooper swans have some branched pores)	Anatidae
3(a)(ii) *Pores unbranched; inorganic capping material*	
Pelicans and allies	Pelecaniformes (implied from Tullett, Board *et al.*, 1976) Except perhaps Tropic birds (Phaethontidae) (Tyler, 1969*a*).
Perhaps grebes	Podicipediformes
4(a) *Pore canals unbranched; outer orifice reticulate*	
Old world vultures	Possibly all the *Accipitridae* except the Lammergeier and Egyptian vulture.

need to dissipate the terminal velocity of rain drops without flooding the pore canals. It is noteworthy that the majority of eggs (Table I) with capped or reticulate pores are found in open nests. Other functions have been suggested in the course of this study. The eggshells of the gannet and penguin are often heavily soiled and it seems probable that the

capillary porous nature of these shells prevents the soiling material from plugging the lumen of the pore canals in the column layers. Those of the shag become impregnated with fats and the capping material is rubbed off during brooding. In fact, the association of capping material with eggs laid in wet, muddy places or in colonies seems to be one of the principal observations of this study.

The classification of pores proposed in this study does emphasize also that with eggs having capped or covered pores, the embryo is exchanging respiratory gases across a tortuous boundary. Moreover, our demonstration that preening oils should be considered a component of the shell implies that the capped or covered pores provide a means of O_2 exchange at rates optimal for the embryo with a minimizing of water loss.

In this communication, the terms cover and cuticle have not been used because with electron optics it was not possible to distinguish between them. Moreover, until the physiology of their deposition is known in detail and their contribution to the functioning of the shell has been defined, there would appear to be little utility in applying them in a preliminary classification which seeks to stimulate the study of overall shell function.

Sincere thanks are due to all the people who supplied material for use in this study (Table I). Additional thanks are due to Dr H. F. Steedman for his suggestions for the plastic modelling technique and to Dr V. D. Scott for the use of a scanning electron microscope.

REFERENCES

Anon (1972). *Fundamentals of transport phenomena in porous media. Developments in soil science* **2**. London: Elsevier.

Ar, A., Paganelli, C. V., Reeves, R. B., Greene, D. G. & Rahn, H. (1974). The avian egg: water vapor conductance, shell thickness and functional pore area. *Condor* **76:** 153–158.

Becking, J. H. (1975). The ultrastructure of the avian eggshell. *Ibis* **117:** 143–151.

Board, R. G. (1974). Microstructure, water resistance and water repellency of the pigeon eggshell. *Br. Poult. Sci.* **15:** 415–419.

Board, R. G. (1975). The microstructure of the cuticle-less shell of the eggs of the domestic hen. *Br. Poult. Sci.* **16:** 89–91.

Board, R. G. & Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biol. Rev.* **49:** 15–49.

Board, R. G. & Halls, N. A. (1973*a*). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poult. Sci.* **14:** 69–97.

Board, R. G. & Halls, N. A. (1973*b*). Water uptake by eggs of mallard and guinea fowl. *Br. Poult. Sci.* **14:** 311–314.

Board, R. G. & Tullett, S. G. (1975). The pore arrangement in the emu (*Dromaius novaehollandiae*) eggshell as shown by plastic models. *J. Microscopy* **103:** 281–284.

Carter, T. (1971). The hen's egg: variation in tensile strength of shell material and its relationship with shearing strength. *Br. Poult. Sci.* **12:** 57–76.

Erben, H. K. (1970). Ultrastrukturen und Mineralisation rezenter und fossiler Eischalen bei Vogeln und Reptilien. *Biamineralisation Forschungsberite* **1:** 1–66. Stuttgart—New York: F. K. Schattaner.

Everett, D. H. & Stone, F. S. (1958). *The structure and properties of porous materials. Proceedings of the 10th Symposium of the Colston Research Society*, Butterworths: London.

Freeman, B. M. & Vince, M. A. (1974). *Development of the avian embryo*. London: Chapman and Hall.

Harrison, C. (1975). *A field guide to the nests, eggs and nestlings of European birds*. Collins: London.

Kutchai, H. & Steen, J. B. (1971). Permeability of the shell and shell membranes of hen's eggs during development. *Resp. Physiol.* **11:** 265–278.

Lack, D. (1968). *Ecological adaptations for breeding in birds*. London: Chapman and Hall.

Luikov, A. (1966). *Heat and mass transport in capillary-porous bodies*. Pergamon Press: London.

Marshall, W. & Cruickshank, D. B. (1938). The function of the cuticle in relation to the porosity of eggs. *J. agric. Sci.* **28:** 24–42.

Martin, J. T. & Juniper, B. E. (1970). *The cuticles of plants*. Arnold: London.
Nathusius, W. von (1868). Uber die Hullen, welche den Dotter des Vogeleies umgeben. *Z. wiss. Zool.* **18:** 225–270.
Sauer, E. G. F. (1972). Ratite eggshells and phylogenetic questions. *Bonn. zool. Beitr.* **23:** 3–48.
Scheidegger, A. E. (1960). *The physics of flow through porous media*. MacMillan: New York.
Schmidt, W. J. (1962*a*). Liegt der Eischalenkalk der Vögel der submikroskopische Kristallite vor? *Z. Zellforsch. mikrosk. Anat.* **57:** 848–880.
Schmidt, W. J. (1962*b*). Uber die Basalkalotten der Vogeleischale. *J. Orn., Lpz.* **103:** 28–37.
Simons, P. C. M. (1971). Ultrastructure of the hen eggshell and its physiological interpretation. *Commun. Centr. Inst. Poultry Res.* No. 175. Beckbergen: The Netherlands: Het Spelderholt.
Tullett, S. G. (1975). Regulation of avian eggshell porosity. *J. Zool., Lond.* **177:** 339–348.
Tullett, S. G. & Board, R. G. (1976). Oxygen flux across the integument of the avian egg during incubation. *Br. Poult. Sci.* **17:** 441–450.
Tullett. S. G., Board, R. G., Love, G., Perrott, H. R. & Scott, V. D. (1976). Vaterite deposition during eggshell formation in the cormorant, gannet and shag, and in "shell-less" eggs of the domestic fowl. *Acta Zool.* **57:** 79–87.
Tullett, S. G., Lutz, P. L. & Board, R. G. (1975). The fine structure of the pores in the shell of the hen's egg. *Br. Poult. Sci.* **16:** 93–95.
Tyler, C. (1956). Studies on eggshells. VII. Some aspects of structure as shown by plastic models. *J. Sci. Fd Agric.* **7:** 483–496.
Tyler, C. (1964*a*). *Wilhelm von Nathusius on avian eggshells*. The University: Reading.
Tyler, C. (1964*b*). A study of the eggshells of the Anatidae. *Proc. zool. Soc. Lond.* **142:** 547–583.
Tyler, C. (1965). A study of the eggshells of the Sphenisciformes. *J. Zool., Lond.* **147:** 1–19.
Tyler, C. (1966). A study of the eggshells of the Falconiformes. *J. Zool., Lond.* **150:** 413–425.
Tyler, C. (1969*a*). A study of the eggshells of the Gaviiformes, Procellariiformes, Podicipitiformes and Pelecaniformes. *J. Zool., Lond.* **158:** 395–412.
Tyler, C. (1969*b*). Avian eggshells: their structure and characteristics. *Int. Rev. gen. exp. Zool.* **4:** 82–127.
Tyler, C. & Simkiss, K. (1959). A study of the eggshells of ratite birds. *Proc. zool. Soc. Lond.* **133:** 201–243.
Wangensteen, O. D. & Rahn, H. (1971). Respiratory gas exchange by the avian embryo. *Respir. Physiol.* **11:** 31–45.
Wangensteen, O. D., Wilson, D. & Rahn, H. (1971). Diffusion of gases across the shell of the hen's egg. *Respir. Physiol.* **11:** 16–30.

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RESEARCH NOTE

THE FINE STRUCTURE OF THE PORES IN THE SHELL OF THE HEN'S EGG

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1. No fibrous or crystalline material was present in the lumen of pore canals which were exposed by radial fracture and examined with a scanning electron microscope.

2. The walls of the canals were rough, but they did not have a characteristic ultrastructure.

3. The campanulate outer orifice of the pores was plugged with cuticle in which radial cracks formed channels through which the respiratory gases presumably diffuse.

INTRODUCTION

The successful development of the avian embryo is largely dependant upon the interaction of the environment imposed on the nest by the brooding parent (Drent, 1967, 1972) and that obtaining within the egg (Board and Fuller, 1974). This concept focuses attention on the role of the shell. This integument has for long been regarded as the physical boundary of cleidoic eggs and properties such as mechanical strength and functions such as the exchange of respiratory gases have been studied in isolation. If the shell is considered to be the mediating boundary or interface between two environments, then there is a need to correlate the form of the shell with the many functions it has to perform. It has been suggested (Board and Halls, 1973; Board and Fuller, 1974) that the various physiological demands on the shell may well conflict and that in consequence selective pressures have resulted in the form of the shell being an evolutionary compromise. Studies of the shell of pigeon eggs, which have little water resistance through being cuticle-less (Board, 1974), indicate that the shell may be adapted to accommodate the demands of the bulk environment, in this case freedom from wetting by rain, to which the eggs are exposed before the onset of continuous brooding.

Although the structure of the shell has been studied extensively (Tyler, 1969), it is probable that information about its fine structure may not be accurate simply because the preparation of specimens may have caused distortion. This may well be caused by grinding shells or sectioning those which have been embedded after fixation and decalcification. It was surmised that distortions would be minimal if

radially fractured shells were merely coated with gold : palladium before examination in a scanning electron microscope. This note presents the observations made in a study of the fine structure of the pores in the shell of the hen's egg.

MATERIALS AND METHODS

The eggs were obtained from a commercial flock kept in batteries and fed on a proprietary diet. They were examined within a few hours to 3 d of oviposition. Some were examined after 40 d storage at room temperature in dessicators containing either anhydrous calcium chloride or water.

Small pieces of shell were fractured so that radial sections could be examined. The shells were either at room temperature or at that of liquid nitrogen at the time of fracture. The shell fragments were cemented (Colloidal Silver, Acheson Colloids Company, Plymouth) on to aluminium stubs so that the tangential axis of the shell was at right angles to the surface of the stub. The shells were plated under vacuum with gold : palladium and examined with a scanning electron microscope (SEM; Stereoscan S4, Cambridge Instrument Co. Ltd) and photographs taken with an accelerating voltage of 10 kV.

RESULTS

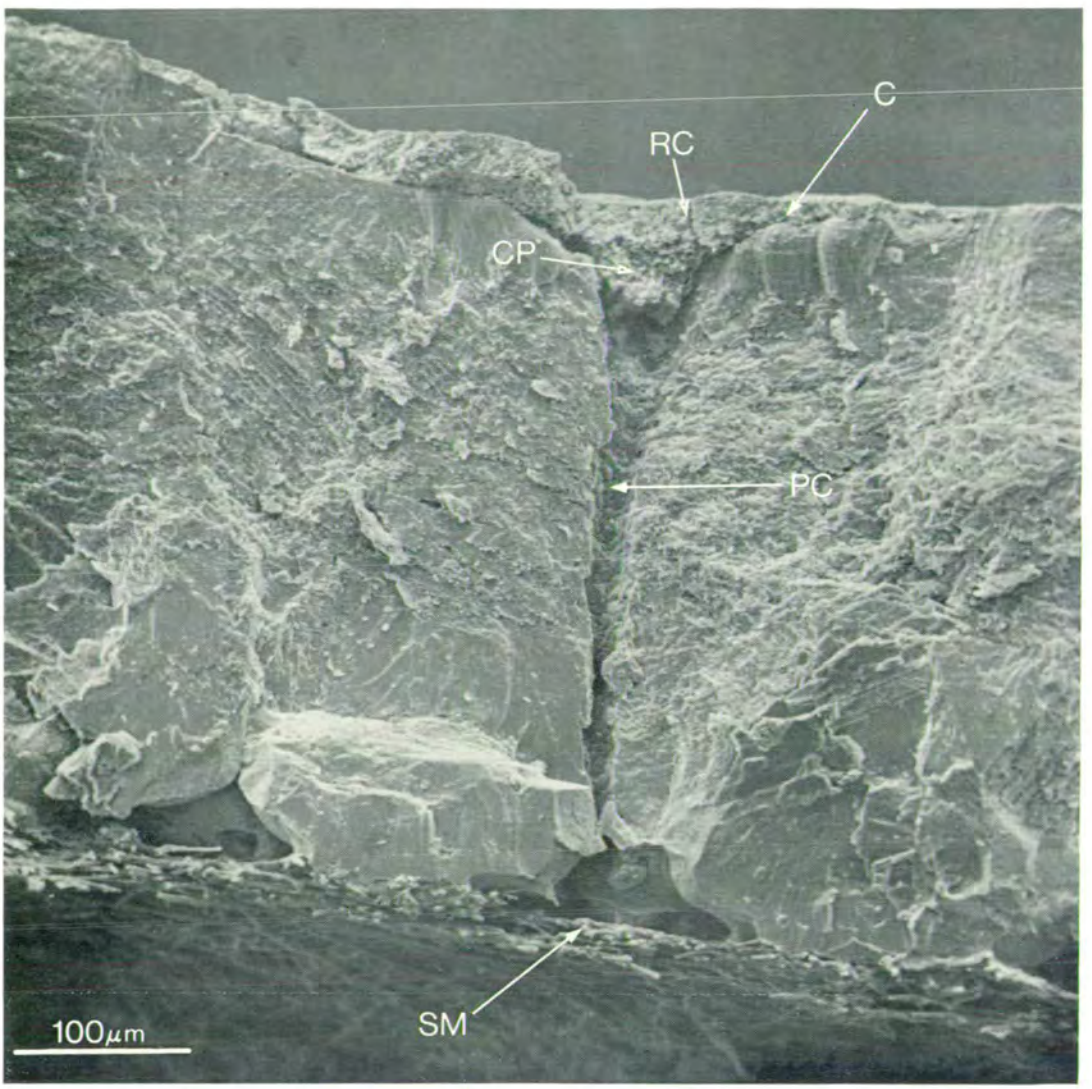
Plate-Fig. 1 shows a pore traversing the face of a radially fractured shell. With brown shells, the outer surface of the column layer was covered with a pavement of cuticle. There was no demonstrable difference in cuticles which had been fractured at room temperature and those which had been fractured immediately following immersion in liquid nitrogen. This suggests that the cuticle or bloom is a rigid, non-flexible coating on the shell. The cuticle extended down the campanulate orifice of the pore thereby forming a crude plug in the shape of a blunt cone. The plug was seen also in shells which had been stored for 40 d at room temperature. White-shelled eggs were covered with a relatively thin cuticle which formed only a small plug in the pore orifice.

The cuticular plugs were fissured radially. These cracks appeared to be the only direct path along which respiratory gases can enter or leave the lumen of the pore canals. The latter was for most of its length free of fibrous or crystalline material and there was no obstruction at the orifice of the opening in the cone layer. With some pores, there was crystalline material protruding into the lumen immediately below the cuticular plug. We were unable to deduce whether it represented fragments deposited during fracture of the shells. If the former were true, then there are still spaces through which gases could be expected to diffuse.

As suggested by Schmidt (1966), the pore canals appeared to be bounded by columns of calcite which under high magnification did not have a characteristic ultrastructure.

DISCUSSION

From their studies, Board and Halls (1973) concluded that the cuticle of the shell of the hen's egg played an important role in waterproofing. Their assumption



PLATE–FIG. 1.—The edge of a piece of radially fractured brown shell showing the cuticle plug (CP) and a radial crack (RC) associated with it, the pore canals (PC), cuticle (C) and shell membranes (SM).

that the shells of naturally cuticle-less eggs were prone to flooding because the pores were uncapped has been supported (Board, 1975) by examination of such shells with a scanning electron microscope. Investigations of pigeon eggs (Board, 1974)—these do not have cuticle—led to the suggestion that the blanket term, waterproofing should not be applied to the shell. The latter's interaction with water may well be considered as either water repellency or water resistance. The present study indicated that the cuticular plugs are well adapted to provide water resistance to the shell of the hen's eggs which are exposed to hydrostatic pressures. In nature, there is presumably a need for eggs in exposed nests to have some means whereby the kinetic energy of rain drops are dissipated without water penetrating the pore canals. A similar situation obtains presumably in egg washing machines which clean or rinse by sprays and there is presumably a requirement to ensure that the force generated by the sprays is below the level at which the shells' resistance would be overcome.

This study has shown that cracks in the cuticular plugs provide channels for the diffusion of respiratory gases. Although the microstructure of the cuticle may change during storage (Simons, 1971), it would not appear to lose its efficiency as a plug of the pore orifice. Thus in studies of gaseous diffusion across the shell covered with cuticle, (Wangansteen and Rahn, 1970-71; Wangansteen *et al.*, 1970-71), attention must be focused on the total area of the tangential cross sections of the cracks in the cuticular plug rather than on the area of the pores *per se*.

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One of us (S. G. T.) wish to thank the British Egg Marketing Board Authority for a Scholarship. We wish to thank Dr V. D. Scott for permission to use the SEM and Mr H. R. Perrott for supervising the preparation and examination of specimens.

REFERENCES

Board, R. G. (1974). Microstructure, water resistance and water repellency of the pigeon egg shell. *Br. Poult. Sci.*, **15**: 415-419.

Board, R. G. (1975). The microstructure of the cuticle-less shell of the eggs of the domestic hen. *Br. Poult. Sci.*, **16**: 89-91.

Board, R. G. and Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biol. Rev.*, **49**: 15-49.

Board, R. G. and Halls, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poult. Sci.*, **14**: 69-97.

Drent, R. H. (1967). Functional aspects of incubation in the herring gull (*Larus argentatus* Pont.). *Ph.D. thesis, Rijksuniversiteit te Groningen.*

Drent, R. H. (1972). Adaptive aspects of the physiology of incubation. *Proceedings of the XVth International Ornithological Congress*, 254-280.

Schmidt, W. J. (1966). Das Verwachsen der Calcosphariten bei der Schalenbildung des Vageleies. *Z. Morph. Ökol. Tiere*, **56**: 252-258.

Simons, P. C. M. (1971). Ultrastructure of the hen egg shell and its physiological interpretation. *Central Institute for Poultry Research "Het Spelderholt" Beekbergen, The Netherlands*, **175**.

Tyler, C. (1969). Avian egg shells: their structure and characteristics. *Int. Rev. gen. exp. Zool.*, **4**: 82-127.

Wangansteen, O. D. and Rahn, H. (1970/71). Respiratory gas exchange by the avian embryo. *Respir. Physiol.*, **11**: 31-45.

Wangansteen, O. D., Wilson, D. and Rahn, H. (1970/71). Diffusion of gases across the shell of the hen's egg. *Respir. Physiol.*, **11**: 16-30.

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Short Technical Note

The pore arrangement in the emu (*Dromaius novæhollandiæ*) eggshell as shown by plastic models

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SUMMARY

Pores arising in the cone layer merge with a plexus of tubules running just under and parallel to the outer surface of the emu eggshell. The outer surface of the shell is irregular due to dark green crystalline material arranged as flat domes. The plexus of tubules vent to the ambient environment *via* short pores opening in the valleys between these domes.

INTRODUCTION

Studies with the light microscope have shown that pores originating in the cone layer terminate some 100–200 μm beneath the outer surface of the shell of emu eggs (Tyler, 1956; Tyler & Simkiss, 1959). They end in a stratum which these authors have referred to as resistant, because of its recalcitrance when embedded in plastic to attack by acid, or fibrous because of its appearance in ground sections viewed with the light microscope. Unlike the other strata of the emu eggshell, this fibrous stratum has been shown (Simons, 1971) in studies with a scanning electron microscope (SEM) to contain large spaces. The available evidence does not permit a mental picture to be readily formed of the means whereby the lumen of the pores is vented to the outside of the shell. This communication presents the results of a study in which a scanning electron microscope and a plastic modelling technique were used to examine the arrangement of the pores in the emu eggshell.

MATERIALS AND METHODS

The eggs

These were infertile and had rotted during incubation. They were laid by emus at Whipsnade Zoo.

Preparation of shell

Pieces of shell were washed in running tap water or scoured for 5 min in boiling NaOH (2·5% w/v). A scanning electron microscope (SEM; Stereoscan S4; Cambridge Scientific Instruments Ltd) was used to study the general arrangement of pores in the radial face of pieces of shell which had been cemented (DAG 915, Acheson Colloid Company, Prince Rock, Plymouth) to aluminium

stubs and coated under vacuum with gold:palladium. An accelerating voltage of 10 kV was used.

Plastic models

In the past 5 years we have had many failures with the plastic modelling technique of Tyler (1956) due to erratic or incomplete polymerization of methacrylate. We have not experienced such problems when using a plastic designed for the amateur modeller (Plasticraft, Turner Research Ltd, Leeds). Pieces of dry, scoured shell were dipped in ethyl acetate and immersed in Plasticraft plastic

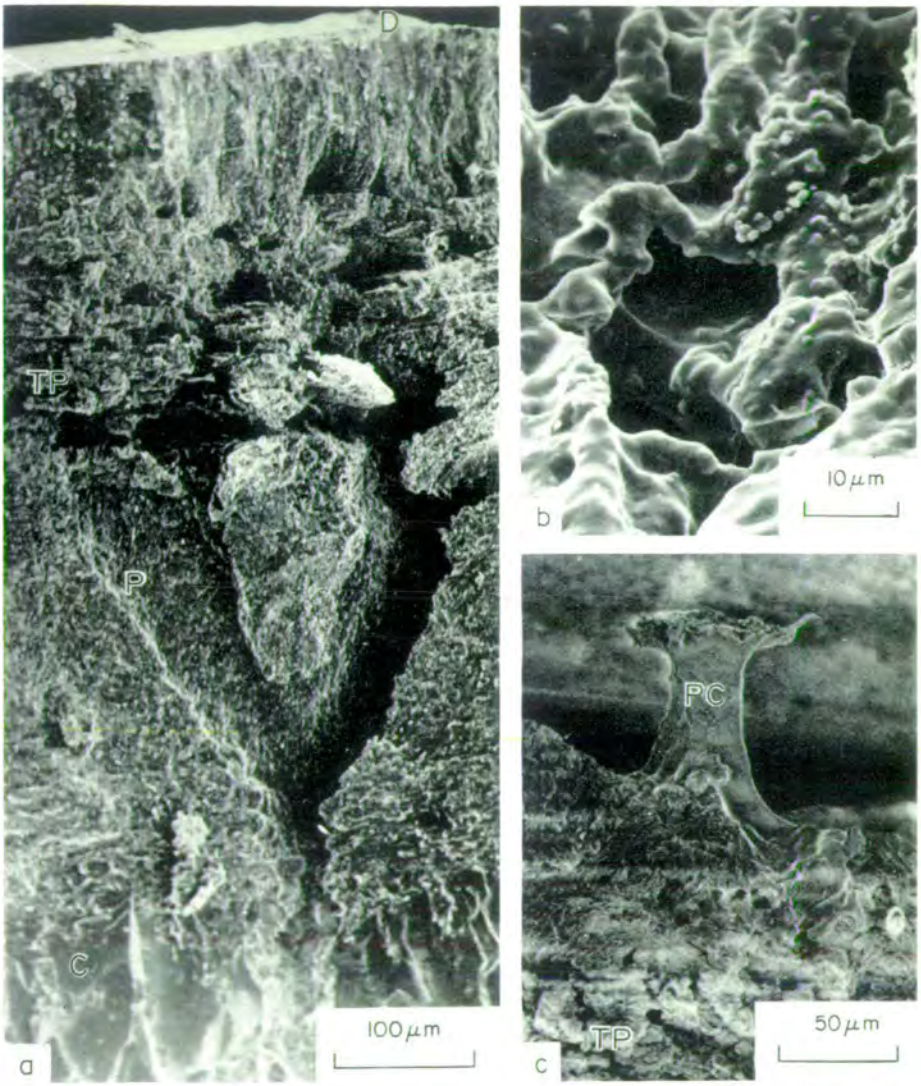

Fig. 1. Emu eggshell. (a) Pore (P) arising in cone layer (C) branches and enters tubule plexus (TP) beneath a dome (D) (× 172). (b) Plastic cast of tubule plexus (× 360). (c) Plastic cast of short pore (PC) through which the tubule plexus (TP) vents to the environment (× 292).

containing catalyst. The shells were infiltrated with plastic by repeatedly drawing and releasing suddenly a vacuum. After polymerization, the plastic was cut with a small hacksaw so that an edge or an outer or inner surface of the shell was exposed. The exposed surface was freed by grinding with 'wet and dry' emery cloth. The shell was decalcified with concentrated hydrochloric acid, washed in 10% (w/v) NaOH and rinsed with tap water. To avoid excessive charging, plastic casts cemented to aluminium stubs were coated under vacuum with carbon and then gold:palladium. They were examined on a SEM with an accelerating voltage of 10 kV.

RESULTS

In SEM studies of the radial face of pieces of emu shell, the pores arising in the cone layer were seen to end at a stratum consisting of loosely packed slabs of crystalline material (Fig. 1a). The outer surface of the stratum was covered by flat domes having an irregular distribution. The crystals in the domes were arranged in columns whose long axes were radial to the surface of the shell. The pores tended to terminate beneath the apogee of the domes.

In studies with a dissecting microscope of tinted plastic models of pieces of shells the inner surface of which had been exposed to concentrated HCl, the plastic casts of the pore canals were seen to fuse with a sheet of plastic running parallel to the cast of the outer surface of the shell. This sheet had a textured appearance and was broken easily when teased with dissecting needles. With the SEM, the sheet was found to be a net of plastic (Fig. 1b) from the outer surface of which short columns of plastic fused with the cast of the outer surface of the shell (Fig. 1c).

DISCUSSION

This study has shown that the lumen of the pores arising in the cone layer vent into a plexus of tubules which are arranged as a net running parallel to the surface of the shell. The net is connected *via* short tubes to valleys between the domes on the surface of the shell. When the layer of the shell containing this net was examined with magnification up to 20 K on the SEM, there was no feature which merited the description, fibrous, a term applied by Tyler & Simkiss (1959) to its appearance in thin sections viewed with the light microscope. The interpretation accorded their alternative term, resistant layer, has to be reconsidered in the light of this study. Acid resistance was not a characteristic of the stratum in which the major pores terminated, the plexus being endowed with this property only when plastic infiltrated the spaces between the slabs of crystalline material.

During incubation, therefore, the embryo will exchange respiratory gases with an atmosphere isolated in the plexus of tubules and the latter will exchange *via* short tubes with the ambient atmosphere. This arrangement contrasts with that of the pigeon egg (Board, 1974), for example, where the lumen of the pores originating in the cone layer vent directly to the outside of the shell.

ACKNOWLEDGMENTS

We would like to express our gratitude to Mr P. J. Olney who kindly supplied the eggshells for this study. Thanks are also due to Dr V. D. Scott for the use of a scanning electron microscope and to Mr H. R. Perrot for assistance with the preparation of material for same.

References

Board, R.G. (1974) Microstructure, water resistance and water repellency of the pigeon eggshell. *Br. Poult. Sci.* **15**, 415.

Simons, P.C.M. (1971) *Ultrastructure of the hen eggshell and its physiological interpretation.* Communication No. 175, Central Institute for Poultry Research. 'Het Spelderholt', Beekbergen, The Netherlands.

Tyler, C. (1956) Studies on eggshells. VII. Some aspects of structure as shown by plastic models. *J. Sci. Food Agric.* **7**, 483.

Tyler, C. & Simkiss, K. (1959) A study of the eggshells of ratite birds. *Proc. Zool. Soc. Lond.* **133**, 201.

Vaterite Deposition During Eggshell Formation in the Cormorant, Gannet and Shag, and in 'Shell-less' Eggs of the Domestic Fowl.

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Abstract

The eggshells of the cormorant (*Phalacrocorax carbo*), domestic fowl (*Gallus domesticus*), gannet (*Sula bassana*), guinea fowl (*Numida meleagris*), greater flamingo (*Phoenicopterus ruber*), and shag (*Phalacrocorax aristotelis*) have been separated into two groups on the basis of the composition of their outer stratum. In the domestic fowl, guinea fowl and greater flamingo the outer stratum is an organic cuticle while in the sea-birds it is an inorganic cover rich in vaterite. The calcareous deposits on the membranes of eggs of the domestic fowl which are shell-less at oviposition have been shown to consist essentially of the vaterite form of calcium carbonate. Reasons for the occurrence of this polymorph of calcium carbonate are discussed with relation to the physiology of the birds.

Introduction

The avian eggshell is composed essentially of calcium carbonate in the form of calcite columns. In the case of the domestic fowl the true shell is covered externally by a thin organic cuticle. This cuticle has a vesicular structure (Simons and Wiertz 1966) and consists of protein, fat and polysaccharides or sugars (Tyler and Simkiss 1958, Cooke and Balch 1970, Wedral et al. 1974). In the eggs of birds belonging to the Phalacrocoridae and Podicepidae (Tyler 1966) the external layer has a chalky appearance, is rich in inorganic material and is more appropriately termed a cover (after Schmidt 1958) rather than a cuticle. Despite these observations no reasons have been given for these differences in structure nor has any suggestion been given of their role in nature.

This paper presents results obtained during a survey of avian eggshells. A detailed study has been carried out using scanning electron microscopy, X-ray diffraction and electron probe microanalysis which has provided new information on the morphology, crystal structure and chemical composition of various types of eggshell. It is shown that the formation of the two basic types of eggshell discussed may be related to the physiology and life-style of the bird. A model is proposed for the formation of the normal eggshell in the domestic fowl and an explanation offered to account for eggs which are shell-less at oviposition.

Materials and Methods

Sources of eggs. Fragments of cormorant eggshell were obtained from Regent's Park; domestic fowl eggs, some of which were shell-less at oviposition, were from commercial laying stock; fragments of gannet eggshell were from Monk's Wood Experimental Station, Abbots Ripton; greater flamingo eggshells from The Wildfowl Trust, Slimbridge; guinea fowl eggs from birds kept in cages or free range and fragments of shag eggshells from Bardsey Island Bird Observatory.

Methods of examination. (a) Scanning electron microscopy: Outer surfaces and sections of radially fractured eggshells were examined. For this purpose specimens of shell were cemented onto aluminium stubs with DAG 915 (Acheson Colloids Co., Prince Rock, Plymouth) and coated under vacuum with a thin (30 nm thickness) layer of gold: palladium alloy. A Stereoscan S4 (Cambridge Scientific Instruments Ltd.) operating at an accelerating voltage of 10 kV was used.

(b) Electron probe microanalysis: Pieces of eggshell were embedded in Plasticraft (Turner Research Ltd., Leeds) using a vacuum impregnation technique to ensure complete infiltration of the shell (Board & Tullett, 1975). Radial sections through the shell were prepared by polishing on successively finer grades of diamond paste down to a 1 μm finish. They were coated under vacuum with a thin layer (30 nm thickness) of gold and examined in a JEOL JXA-50A electron probe microanalyser.

c) Chemical assay: The phosporus content of the different eggshell samples was calculated from phosphate determinations obtained using a modified Fiske-Subbarow (1925) method. Samples of organic cuticles (10 mg), crystalline covers (50 mg) and outer shell (50 mg) were obtained by scraping the eggshell with a scalpel. These samples as well as the inner shell (100 mg) remaining from such a process and whole true shells (100 mg) were analysed. The material was dissolved in 0.5 ml HCl (A.R.) and maintained at 100°C for 5 min. The solution was made up to 6 ml with water and a dilution series prepared from this for analysis. Phosphate was assayed by addition of 1 ml 2.5 % ammonium molybdate followed by 1 ml reducing agent (3 % $NaHSO_3$, 1 % Elon (Kodak Ltd, London)) and reading, after 20 min., the optical density at 660 nm.

(d) Flame photometry and atomic absorptiometry: Samples of eggshell were dissolved as above and made up to a volume of 10 ml. Sodium and potassium levels were measured using a flame photometer (E.E.L. Instruments, Essex) and magnesium using an atomic absorptiometer (Unicam SP 90A Series 2, Pye Instruments, Cambridge).

(e) X-ray diffraction: The crystal structure of the shell specimens was examined by the Debye-Scherrer method using copper K_α-radiation at 40 kV and 20 m. amp with a nickel filter.

Results

Scanning electron micrographs taken from a radial section through the eggshell of a greater flamingo (total thickness 0.70 mm) are illustrated in Figs. 1 a and b. These show an abrupt change from the columnar crystals of the true shell to a vesicular cuticle of about 0.07 mm thickness. The columnar crystals were found by X-ray diffraction to be calcite. The material forming the cuticle had a chalky appearance. However, it gave no X-ray diffraction pattern and, moreover, dissolved in boiling sodium hydroxide (5 % w/v) which suggested that it was organic in nature.

Electron probe microanalysis on polished radial sections of the greater flamingo eggshell showed a marked increase in the phosphorus level from the shell (0.15 wt%) to the cuticle (4.0 wt%) as illustrated in the line scan in Fig. 1 c. The cuticle was estimated to contain around 15 % by weight of calcium calculated on the assumption that the shell ($CaCO_3$) contained about 36 wt%. A number of other elements including chlorine, magnesium, potassium and sodium were detected but these were in much lower concentrations (usually <0.1 wt%) and, due to the nature of the samples, concentration changes across the section were difficult to ascertain. A small increase in the sulphur level was detected from the outer shell (0.1 wt%) to the cuticle (0.5 wt%). Chemical analyses confirmed these data and indicated a marked difference in the phosphorus content between the shell and cuticle (Table 1).

The shell of domestic fowl eggs, which are known to have an organic cuticle (Tyler and Simkiss 1958, Cooke and Balch 1970, Wedral et al. 1974), and those of the guinea fowl gave, apart from the absence of calcium in the cuticle, essentially similar results to those of the greater flamingo (Table 1).

Scanning electron micrographs taken from a radial section through the eggshell of a gannet (total thickness 0.45 mm) are illustrated in Figs. 1 d and e. An abrupt change from columnar crystals to a spherulitic cover of about 0.06 mm thickness can be seen. A notable difference between Figs. 1 e and b is that the particles forming the cover are larger (0.35—3.40 μm cf. 0.01—0.05 μm diameter) than those of the cuticle. In the gannet eggshell the columnar crystals were found by X-ray diffraction to be calcite (Fig. 3 a). The cover gave a clear X-ray diffraction pattern (Fig. 3 b) and was identified with vaterite, the third polymorph of calcium carbonate.

Electron probe microanalysis on polished sections of gannet eggshells showed there was a much smaller difference in phosphorus levels between the shell (<0.1 wt%) and cover (0.4 wt%) than with eggs having a cuticle. Traces of chlorine, magnesium, potassium, sodium and sulphur were found (<0.1 wt%), with indications of slightly more chlorine, magnesium and sulphur towards the outer layers. The above data are in general agreement with results obtained by chemical assay (Table 1).

The eggs of two other sea-birds, cormorant and shag, were similar to that of the gannet in having a crystalline cover of vaterite over the true shell of calcite. Certain of the larger spheres possessed a dimpled appearance as shown in

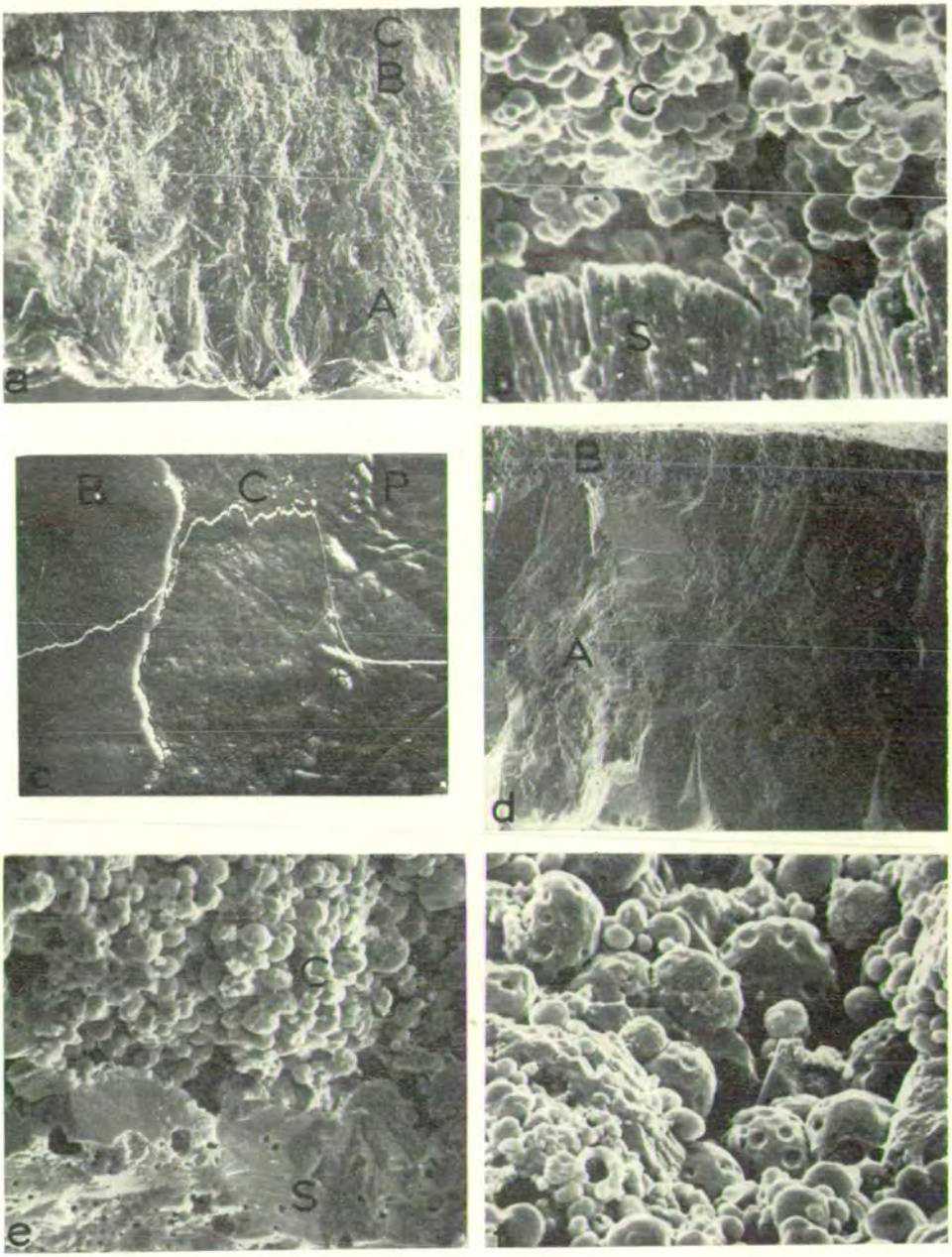


Fig. 1. a. Scanning electron micrograph (SEM) of a radially fractured greater flamingo eggshell showing the regions (*A* inner shell, *B* outer shell, *C* cuticle) analysed in the study. *b.* High magnification of 1 a showing abrupt change from columnar crystals of true shell (*S*) to the vesicular organic cuticle. (*C*). *c.* Line scan for phosphorus across the outer strata of a greater flamingo eggshell. This micrograph shows the increasing level of phosphorus from the outer shell (*B*) to the cuticle (*C*) and its absence in the embedding plastic (*P*). The horizontal line indicates the location of the scan on the specimen. *d.* SEM of a radially fractured gannet eggshell showing the regions (*A* true shell, *B* cover) analysed in the study. *e.* High magnification of 1 d showing change from the true shell (*S*) to the cover (*C*). The outer shell layer is less ordered compared to the greater flamingo and many pit-like holes are present in the shell—a feature of many sea-bird eggs we have examined. *f.* Outer surface of a shag eggshell showing the spherulitic deposits forming the cover. *a* 75×, *b* 3575×, *c* 250×, *d* 125×, *e* 1435×, *f* 3260×.

Table 1. *Elemental composition (wt%) of avian eggshells*

Eggs with an organic cuticle					Eggs with a vaterite cover			
Species	Element	A	B	C	Species	Element	A	B
Domestic Fowl	P	0.08—0.09	1.60—2.80	2.50—2.80	Cormorant	P	0.07—0.09	0.50—0.55
	Mg	0.43—0.48	1.50	1.63—1.67		Mg	0.09	0.14
	Na	0.04	0.04	0.02		Na	0.05	0.04
	K	0.01	0.03	0.02		K	0.03	0.08
Greater Flamingo	P	0.13—0.15	1.70—2.30	3.00—4.00	Gannet	P	0.05—0.06	0.35—0.40
	Mg	0.16—0.18		0.13—0.17		Mg	0.15	0.10
	Na	0.05		0.02		Na	0.05	0.04
	K	0.02		0.30		K	0.03	0.08
Guinea Fowl	P	0.04—0.08	0.70—2.30	5.00—6.40	Shag	P	0.04—0.05	0.35—0.40
	Mg	0.42—0.45	0.70—0.75	2.19—2.80		Mg	0.14	0.09
	Na	0.05	0.06	0.01		Na	0.05	0.05
	K	0.02	0.03	0.20		K	0.03	0.08
					Shell-less Domestic Fowl	P	—	0.11—0.13
						Mg	—	0.24
						Na	—	0.05
						K	—	0.08

Note: Itoh and Hatano (1964) have shown increases in the P and Mg levels between the inner and outer shell of the eggs of domestic fowl. The outer shell values quoted in this table also reflect the presence of a "cuticular" component as, owing to the nature of preparation of the samples, pore plugs would be present. A, B, C, see Fig. 1.

Fig. 1 f. The chemical composition data were also similar to those obtained for the gannet eggshell (Table 1).

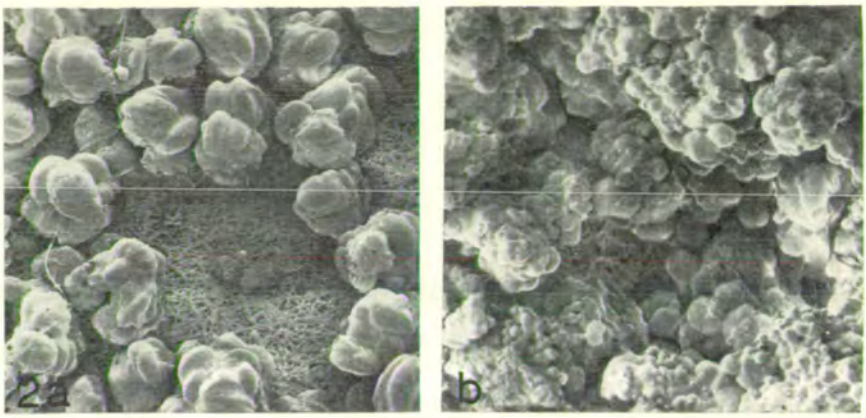
A scanning electron micrograph of the surface of a domestic fowl egg that was shell-less at oviposition is illustrated in Fig. 2 a and shows the spherular nature of the deposits on the outer shell membrane. Eggs on which the deposits were thicker (Fig. 2 b) resembled surface views of the gannet eggshell. The deposits on shell-less eggs were found to consist essentially of vaterite (Fig. 3 c). Chemical analysis showed that the sodium levels were similar, magnesium levels lower and phosphorus and potassium levels slightly higher than those found in the inner shell of normal eggs of the domestic fowl. Hence, these deposits resemble more closely the cover of the sea-birds studied than the normal shell of the domestic fowl.

An Aylesbury duck egg which was formed normally but due to antiperistalsis had been moved back up the oviduct was found to be overlain with albumen and two shell membranes. Calcium carbonate was crystallised upon the outer shell membrane as a mixture of calcite and vaterite (Fig. 3 d).

Discussion

The present study on the eggshells of the domestic fowl, guinea fowl, greater flamingo, cormorant, gannet and shag has shown that they may be separated into two categories. The eggs of the domestic fowl, guinea fowl and greater flamingo consist of the true shell of calcite covered on its external surface with cuticle. The eggs of the sea-birds comprise a true shell of calcite covered externally by a layer of vaterite.

The work has also demonstrated that the calcium carbonate laid down on the outer shell membrane of domestic fowl eggs which are shell-less at oviposition consist essentially of particles of vaterite. Hence, these deposits resemble the cover of certain sea-birds rather than the normal mammillary knobs laid down during the formation of domestic fowl eggshells which have been shown by Fujii and Tamura (1971). It is noteworthy that as long ago as 1869 von Nathusius had shown



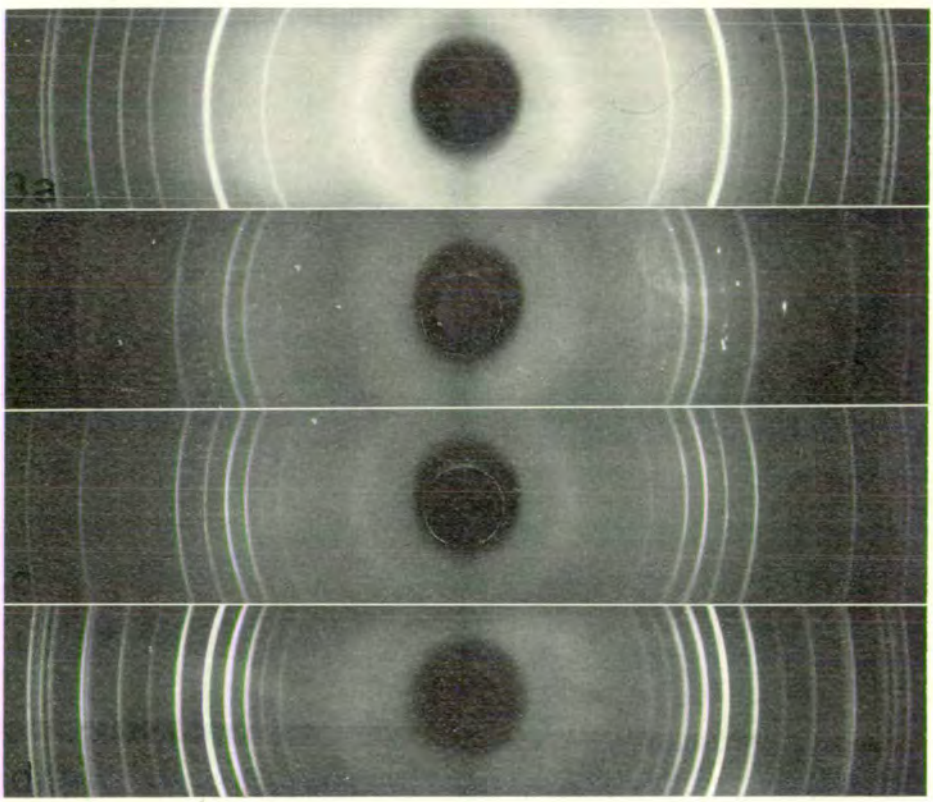


Fig. 2. a. Surface view of a "shell-less" domestic fowl egg. A little crystallisation has occurred forming unusual nodular mamillary knobs on the surface of the outer shell membrane. *b.* Surface view of a "shell-less" domestic fowl egg with more advanced crystallisation. *a* 125×, *b* 310×.

Fig. 3. X-ray diffraction patterns from: *a.* Powdered inner shell layers from a gannet egg. The pattern is characteristic of calcite. *b.* Powdered cover from a gannet eggshell. This pattern is that of vaterite. The prominent calcite line from Fig. 3 a is also represented as a series of spots indicating that a little shell calcite must have been removed as the cover was scraped from the egg. *c.* Powdered deposits from a "shell-less" domestic fowl egg. Traces of calcite are present but the predominant pattern is of vaterite. *d.* Pattern for the outer soft shell of a "double-shelled" Aylesbury duck egg. This "shell" is crystallised as a mixture of calcite and vaterite.

an unusual mammillary structure in a soft-shelled turkey egg. This consisted of nodules of shell material resting on the outer shell membrane. Since previous work (Buckner et al. 1922) has shown that lack of calcium is not the causative factor in the production of shell-less eggs some other change at the crystallisation site must be responsible. The present evidence indicates that alterations in the phosphorus levels, present as phosphates derived from the blood, may cause the change from calcite to vaterite in eggshells having a crystalline cover and terminate eggshell formation in eggs having an organic cuticle. The latter possibility has been intimated by Simkiss (1964) who discussed phosphates as poisons of calcification and by Simons (1971) who suggested they may help terminate eggshell formation.

The avian eggshell is elaborated from raw materials removed from the blood at the shell gland. The calcium and carbonate ions are assembled sandwich style. It is postulated that there is an equilibrium between phosphates that are bound to carriers in the blood and do not cross the shell gland wall and phosphates which are free to cross the wall. Phosphate which enters the shell gland may then substitute for carbonate ions in the calcite lattice. It is suggested that while a small amount of phosphate (equivalent to 0.1 wt% phosphorus) can be incorporated into the calcite lattice, too much may render this structure unstable. According to Raistrick (1949), phosphate ions may remove the electrostatic potential for the absorption of a further layer of calcium ions.

In the case of the domestic fowl, guinea fowl and greater flamingo the phosphate levels increase to such an extent (incorporation rate of above about 2.5 wt%) as to terminate all crystallisation. The organic cuticle is then added.

In the case of the sea-birds the phosphorus content was 0.33—0.55 wt% in the cover compared with <0.1 wt% in the calcitic shell. The phosphorus level of 0.33—0.55 wt% is not sufficiently high to terminate crystallisation but incorporation of this amount of phosphorus as phosphates in the calcium carbonate lattice results in vaterite becoming the stable structure rather than calcite. Vaterite has only previously been found once to our knowledge in eggshells (Gould 1972) and only rarely occurs in biological systems, for example, in biliary calculi (Bogren and Larsson 1963), on experimental regeneration of the shells of certain molluscs and the alga *Coccolithus huxleyi* (Wilbur and Watabe 1963), as an accompanying mineral in calcite kidney stones in horses (Gruenberg 1971), and in the reproductive systems of certain snails (Watabe et al. 1973).

The vaterite crystals are seen in this study to adopt a spherulitic form. This morphology suggests that fresh calcium carbonate crystals are being nucleated but, probably due to the level of phosphate (equivalent to 0.33—0.55 wt%), are not allowed to develop into large crystals characteristic of the growth of the inner layers of calcite. It is not possible at this stage in the work to say what material forms the nucleation sites but it is probably connected with the natural secretions of the oviduct.

The next question to be answered is why, in the final stages of shell formation, higher levels of phosphate are present in the domestic fowl, guinea fowl and greater flamingo than in the seabirds? The sudden increase in the phosphorus level from the outer shell to the cuticle in the eggs of the domestic fowl, guinea fowl and greater flamingo probably reflects a switch from crystallisation of the true shell to the secretion of the organic cuticle within the shell gland. The situation in the sea-birds is more complex and the smaller rise in phosphorus content between the shell and outer cover must be associated with a change in the source of calcium for eggshell crystallisation from one having a high Ca:P ratio to one having a low Ca:P ratio. Indeed, two types of calcium reserve have been identified in the domestic fowl (Common and Hale 1942, Black and Tyler 1944) which may be used when the dietary intake is insufficient to provide all the shell calcium. One is a readily mobilised reserve which these authors suggest is probably used at the beginning of shell formation and has a relatively high Ca:P ratio while the other is a less readily mobilised reserve which is used towards the end of shell formation and has a low Ca:P ratio. It is interesting to speculate what these two reserves are and what their relevance is to egg production in the birds studied.

It is known that calcium for eggshell formation is drawn from a special reserve known as medullary bone laid down in the marrow cavities of the limb bones at the onset of the reproductive period. The breakdown of medullary bone results in a concommitant release of phosphate which is excreted (see Simkiss 1967). Thus several, not mutually exclusive, possibilities present themselves towards the end of shell formation. First, the diet and/or the initial medullary bone store (calcium store 1) is sufficient to provide all the calcium for shell formation. Secondly, this store

may become depleted and the bird then has to draw on a calcium store (2) which may be formed either of secondary medullary bone formed during depletion of Store 1, or it may be the skeletal bone itself. In the case of the domestic fowl, guinea fowl and greater flamingo calcium store 1 is presumably sufficient to provide all the shell calcium and the increase in phosphorus towards the end of shell formation reflects, as suggested previously, a switch from crystallisation to cuticle secretion rather than a switch in the source of calcium. Calcium store 2 probably does not become operational in such birds until the final eggs in a protracted clutch.

It is suggested that the birds which have to fly out to sea for food, eg. the cormorant and shag, avoid a large increase in body weight during the breeding season by laying down only a small medullary bone store. The gannet, which plunges into the water for food from heights of 50 feet and more has, among other shock-absorbing features, a strengthened skeleton to withstand impact with the water (Beazley 1974). Here again the scope for medullary bone storage may be limited. Hence, a switch from calcium store 1 to store 2 is likely to be made before shell formation is complete. This switch it is suggested may lead to an increase in the phosphate concentration to levels which favour vaterite rather than calcite formation.

The eggs of the greater flamingo are interesting in two further respects. Firstly, the proposed switch from crystallisation to cuticle secretion does not appear to close down the incorporation of calcium into the shell. Hence, the cuticle contains a high proportion of calcium but, again possibly due to the high phosphate concentrations, this is not crystallised and no X-ray diffraction pattern is obtained from specimens of cuticle. Secondly, the inner shell of the greater flamingo had the highest phosphorus content of any of the eggs studied. This must be related to the fact that the greater flamingo and its close relative the lesser flamingo inhabit eutrophic waters (Brown 1959) which are naturally rich in phosphorus (Middlebrooks et al. 1974) and their diet includes phosphate-rich blue-green algae.

The proposed mechanism of eggshell formation can be extended to explain certain unusual features of eggshell structure, for example, the vaterite form of the calcium deposits laid down on the outer shell membrane of domestic fowl eggs which are shell-less at oviposition. Such eggs occur regularly within laying flocks and observations at a local poultry farm suggest they are laid only a few hours after a normal egg rather than the usual 24.5 hour interval. This suggests that two oocytes may have been ovulated simultaneously or within a short interval. The first receives a normal calcite shell covered with a phosphate-rich cuticle. The second egg enters the shell gland as the phosphate levels are falling after the cuticle secretion for the first egg. The level is, however sufficiently high to impede calcite growth and, in consequence, vaterite becomes the more stable form. Further evidence for this mechanism has been provided by a normal Aylesbury duck egg which just prior to oviposition was moved back up the oviduct. The coating of albumen received by this egg indicates that it moved up as far as the magnum—hence on the basis of figures presented by Gilbert (1971) it would be about three hours before it re-entered the shell gland. This is a longer interval than that occuring between the proposed exit of egg 1 and the entry of egg 2 in the production of shell-less eggs. It would be expected, therefore, that the phosphate levels would have fallen lower than those permitting only vaterite production. Indeed, the membranes of the "outer" egg had received calcareous deposits consisting of a mixture of calcite and vaterite. The formation of weak eggshells may also be explained similarly although in this case it is suggested that the shell gland fluid composition recovers to favour normal calcite deposition upon an abnormal cone layer. This view is supported by the studies of King and Robinson (1972) who published micrographs of weak eggshells showing an unusual cone layer composed of spherular units. Unfortunately, no data as to its chemical composition are available.

The conditions necessary for vaterite formation appear to be present at some time during each egg laying cycle. Of the birds studied, only the sea-birds seem to have made use of this feature to provide a vaterite cover for the egg. This cover must have intrinsic properties favourable to the successful development of the embryo. One possible function that has been suggested (Board et al. 1976) is it helps keep the egg clean.

Vaterite has been identified in eggshells only once previously (Gould 1972) in the case of the brown pelican. Unfortunately whole shells were analysed and the location of the vaterite within the eggshell was therefore not apparent. However, it seems reasonable to assume that the pelicans, which are close allies of the cormorant, gannet and shag, have a similar shell structure to these birds. The fact that a relatively thin shell in

Gould's study had an infinitely high calcite to vaterite ratio (supposedly as a result of the effects of the metabolites of DDT) may indicate that the egg was coverless—a situation akin to cuticle-less eggs in the domestic fowl discussed by Board (1975). One of the functions of the vaterite cover must be as a mechanical defence against microbial penetration of the shell (see Board and Fuller, 1974). Therefore if the coverless state is a result of the effects of the metabolites of persistent pesticides this introduces the question of egg losses through addling in addition to the usual losses through breakage discussed by Ratcliffe (1970).

Acknowledgements

One of us (S.G.T.) would like to thank the British Egg Marketing Board Research and Education Trust for a scholarship. Thanks are due to the S.R.C. for the original provision of a scanning electron microscope and to people who supplied material for study; Mr. and Mrs. H. F. Hoddinott (domestic fowl), Mr. G. Tucker (guinea fowl), Prof. G. V. T. Matthews (greater flamingo), Mr. M. Peacock (shag), Mr. P. J. Olney (cormorant) and Dr. J. L. F. Parslow (gannet). Thanks are also due to Mr. B. Chapman for production of the X-ray diffraction patterns.

References

Beazely, M. 1974. *World Atlas of Birds.* Mitchell Beazely Publishers Ltd., London.

Black, D. J. G., Tyler, C. 1944. Fluctuations in the porosity of eggshells. *Nature,* Lond. *153:* 682—683.

Board, R. G. 1975. The microstructure of the cuticle-less shell of the eggs of the domestic hen. *Br. Poult. Sci. 16:* 89—93.

— *Tullett, S. G.* 1975. The pore arrangement in the emu (*Dromaius novaehollandiae*) as shown by plastic models. *J. Microscopy 103:* 281—284.

— — *Perrott, H. R.* 1976. A classification of pore systems in avian eggshells. *J. Zool. London.*

Bogren, H., Larsson, K. 1963. Crystalline components of biliary calculi. *Scand. J. Clin. Lab. Invest. 15:* 457—462.

Brown, L. H. 1959. *The Mystery of the Flamingoes.* Country Life, London.

Buckner, G. D., Martin, J. H., Pierce, W. C., Peter, A. M. 1922. Calcium in eggshell formation. *J. Biol. Chem. 51:* 51—54.

Common, R. H., Hale, R. W. 1941. Observations on the mineral metabolism of pullets. VI. The mobilization of body calcium for shell formation. *J. Agric. Sci. 31:* 415—437.

Cooke, A. S., Balch, D. A. 1970. Studies of membrane, mammillary cores and cuticle of the hen eggshell. *Br. Poult. Sci. 11:* 345—352.

Fiske, C., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem. 66:* 375—400.

Fujii, S., Tamura, T. 1970. Scanning electron microscopy of shell formation in the hens egg. *J. Fac. Fish. Anim. Husb.,* Hiroshima Univ. *9:* 65—81.

Gilbert, A. B. 1971. Transport of the egg through the oviduct and oviposition. In *Physiology and Biochemistry of the Domestic Fowl.* Vol. 3. (Bell, D. J., Freemen, B. M., eds.), Academic Press, London.

Gould, R. W. 1972. Brown pelican eggshells: X-ray diffraction studies. *Bull. Environ. Cont. and Tox. 8:* 84—88.

Gruenberg, W. 1971. Karbonat-Harnsteine herbivorer Säugetiere. *Zentralbl. Veterinärmed. Reihe A. 18:* 767—823.

Itoh, H., Hatano, T. 1964. Variation of magnesium and phosphorus deposition rates during egg shell formation. *Poult. Sci. 43:* 77—80.

King, N. R., Robinson, D. S. 1972. The use of the scanning electron microscope for comparing the structure of weak and strong egg shells. *J. Microscopy 95:* 437—443.

Middlebrooks, E. J., Falkenborg, D. H., Maloney, T. E. 1974. *Modelling the Eutrophication Process.* Ann Arbor Science Publins, Michigan.

Nathusius, N. von. 1869. Nachtrage zu der Arbeit "Über die Hüllen, welche den Dotter des Vogeleies umgeben". *Z. wiss. Zool. 19:* 322—348.

Raistrick, B. 1949. The influence of foreign ions on crystal growth from solution. I. The stabilization of the supersaturation of calcium carbonate solutions by anions possessing O-P-O-P-O chains. *Disc. Faraday Soc. 5:* 234—237.

Ratcliffe, D. A. 1970. Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some British birds. *J. appl. Ecol. 7:* 67—115.

Schmidt, W. J. 1968. Schleim-, Kalk- und Farbstoff-Ablagerungen auf der Kalkschale von Vogeleiern. *Z. Zellforsch. 47:* 251—268.

Simkiss, K. 1964. Phosphates as crystal poisons of calcification. *Biol. Rev. 39:* 487—505.

— 1971. *Calcium in Reproductive Physiology.* Chapman and Hall Ltd., London.

Simons, P. C. M. 1971. Ultrastructure of the hen eggshell and its physiological interpretation. *Comm. No. 175.* Central Institute for Poultry Research. Beekbergen, the Netherlands.

— *Wiertz, G.* 1966. The ultrastructure of the surface of the cuticle of the hens egg in relation to egg cleaning. *Poultry. 45:* 1153—1162.

Tyler, C. 1969. A study of the egg shell of the

gaviiformes, procellariformes, podicipitoformes and pelicaniformes. *J. Zool. Lond. 158:* 395—412.

— *Simkiss, K.* 1958. Studies on egg shells. VIII. The determination of membrane-, pore- and matrix-protein—a reassessment. *J. Sci. Fd. Agric. 9:* 375—380.

Watabe, N., Meenakshi, V. R., Blackwelder, P. L., Dunkelberger, D. G. 1973. Distribution of vaterite crystals in reproductive systems of ampullarid snails. *Am. Zoolog. 13:* 1334.

Wedral, E. M., Vadehra, D. V., Baker, R. C. 1974. Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of *Gallus gallus. Comp. Biochem. Physiol. 47B:* 631—640.

Wilbur, K. M., Watabe, N. 1963. Experimental studies on calcification in molluscs and the alga *Coccolithus huxleyi. Ann. N. Y. Acad. Sci. 109,* Art 1, 82—112.

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THE PLUGGED PORES OF TINAMOU (TINAMIDAE) AND JAÇANA (JACANIDAE) EGGSHELLS

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To aid comparative studies of shell function, an arbitrary classification of the pore systems in eggshells was proposed by Board *et al.* (1977). Although having a utilitarian objective only, it directed attention at a neglected area of study: the physiological control mechanisms which cause the formation of intricate pore systems during the short period that the egg is in the shell gland. It has been suggested (Tullett 1975, Tullett & Board 1977) that the initiation of pores results from the distribution of the seeding sites of calcification—aggregates of proteins—on the outer shell membranes; the mechanisms that determine their ultimate form are not known.

The present communication describes the pore systems in the eggshells of four species of tinamous (Tinamidae) and a jaçana (Jacanidae). Not only does it amplify the observations made by Tyler & Simkiss (1959) on the tinamou egg, it extends also the classification of pores proposed by Board *et al.* (1977) and poses yet again questions about the means whereby pores are fashioned in the shell gland.

MATERIALS AND METHODS

Eggshells of tinamous were obtained from Dr Lloyd Kiff of the Western Foundation of Vertebrate Zoology, Los Angeles, and Drs U. K. Abbott and C. R. Grau, Davis, California. Those of the Lesser Jaçana *Micropara capensis* were provided by Dr W. Tarboton of South Africa.

Eggshell fragments were examined with the Stereoscan S4 (Cambridge Scientific Instruments Ltd), scanning electron microscope and in a JEOL JXA-50A electron probe microanalyser. For studies of fine structure, an accelerating voltage of 10 kV was used. Eggshells were glued with colloidal silver (DAG 915; Acheson Colloids Company, Princes Rock, Plymouth) on to round aluminium specimen holders. The holders with attached samples were coated *in vacuo* with gold. If not examined immediately, they were kept in a desiccator over silica gel. For analysis with the electron probe microanalyser, radial sections through the shell embedded in Spur resin (Taab, Emmer Green, Reading) were polished with successively finer grades of diamond paste down to a 1 μm finish. The polished surface was coated *in vacuo* with gold. X-ray diffraction of powdered shell was examined by the Debye–Scherrer method using K_a-radiation at 40 kV and 20 m.amp with a nickel filter. This method will detect crystal forms other than calcite only if they constitute more than 2% of the whole.

To remove the material from the outer orifice of the pore canals, fragments of shells were boiled (the eggshells of tinamous for five minutes and those of jaçana for ten) in sodium hydroxide solution (aqueous 5% w/v). After thorough washing in running tap water, the fragments were dried in the laboratory and examined as described above.

OBSERVATIONS

OVERALL STRUCTURE

The general structure of the eggshells of tinamou and jaçana, as seen in radial section, is shown in the stylized line drawings in Figure 1. Two common features are worthy of

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note: first, the trilaminate shell, an easily distinguishable middle section being sandwiched by two thin layers of dense crystalline material, and, second, the outer companulate orifice of the pore canal with its cone-shaped plug. The differences in fine structure are considered in the following sections.

THE TINAMOU EGGSHELL

When examined within a few weeks of being laid, with the scanning electron microscope (SEM), the outer surface of one sample of the porcelain-like eggshell of the Elegant Crested Tinamou *Eudromia elegans* showed no feature other than circular patches (Plate 24a). As was to be expected from the observations of Tyler & Simkiss (1959), the outer part of the pore canal (Plate 24b) was revealed when these patches were removed by boiling sodium hydroxide solution. This treatment diminished the glossiness of the shell but it had no other effect on the appearance of its surface, as judged by examination with the SEM. In contrast to the single fissure in patches on this shell, multiple fissuring was present in patches on older eggshells of this and other tinamous obtained from a museum collection (Plate 25). None the less, such patches were the only notable features on the outer surface of eggshells of Highland Tinamou *Nothocercus bonapartei*, Spotted Nothura *Nothura maculosa* and Red-Winged Tinamou *Rhyncotis rufescens*. We therefore assume that the multiplicity of fissuring was an effect of long storage, and the following description is based on the comparatively fresh eggshells of Elegant Crested Tinamou (obtained from Dr U. K. Abbott).

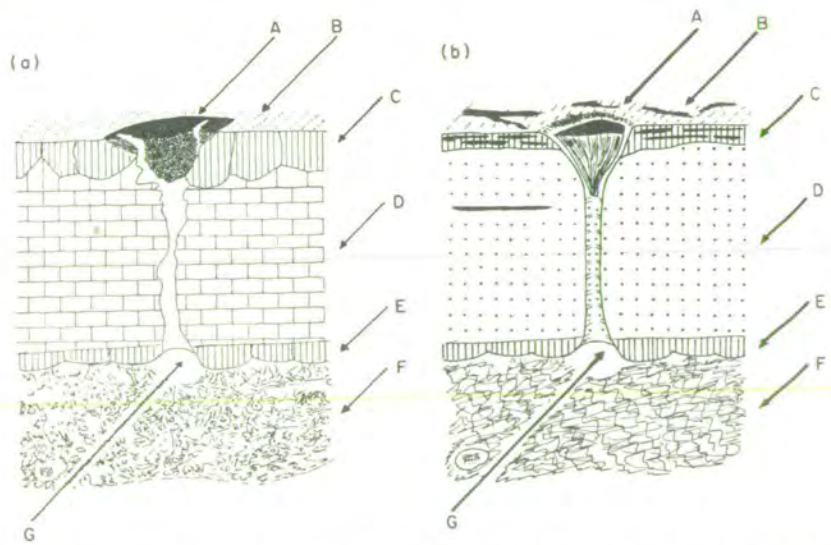


FIGURE 1. Radial section through eggshell of (a) tinamou and (b) jaçana showing pore plug (A), outer surface of shell (B), outer crystalline layer (C), middle stratum of shell (D), inner crystalline layer (E), shell membranes (F) and inner orifice of pore canal (G). Drawn to scale from scanning electron micrographs.

As evident from scanning electron micrographs of radially fractured eggshells (Plate 26), the patches were the relatively smooth outer surfaces of plugs which extended down into the pore canals. The major portion of the plug, as noted by Tyler & Simkiss (1959), was contained in a hole in the dense crystalline material (Plate 26a) on the outer surface of the shell. The plug consisted of spheres (average diameter 0·3 μm, range 0·1–0·5 μm) or spheres and short fibres (Plate 26c). The single fissure shown in Plate 24a was located in the plug; the peripheral portion of the latter was closely associated with the outer crystalline

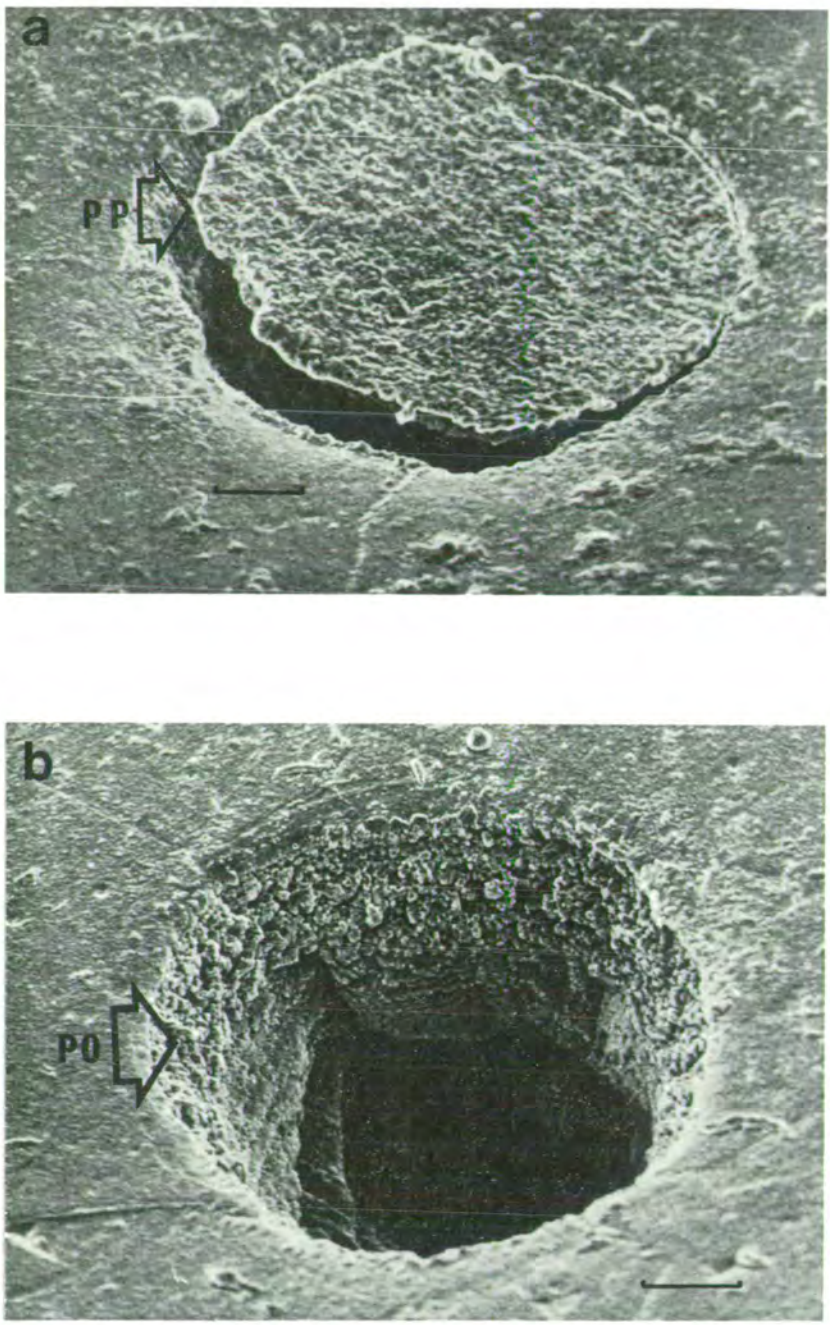


PLATE 24. The surface of (a) the untreated and (b) sodium hydroxide-treated eggshell of the Elegant Crested Tinamou *Eudromia elegans*, origin Davis, California, U.S.A. PP, pore plug and PO, pore orifice. Scanning electron micrograph; scale bar = 10 μm.

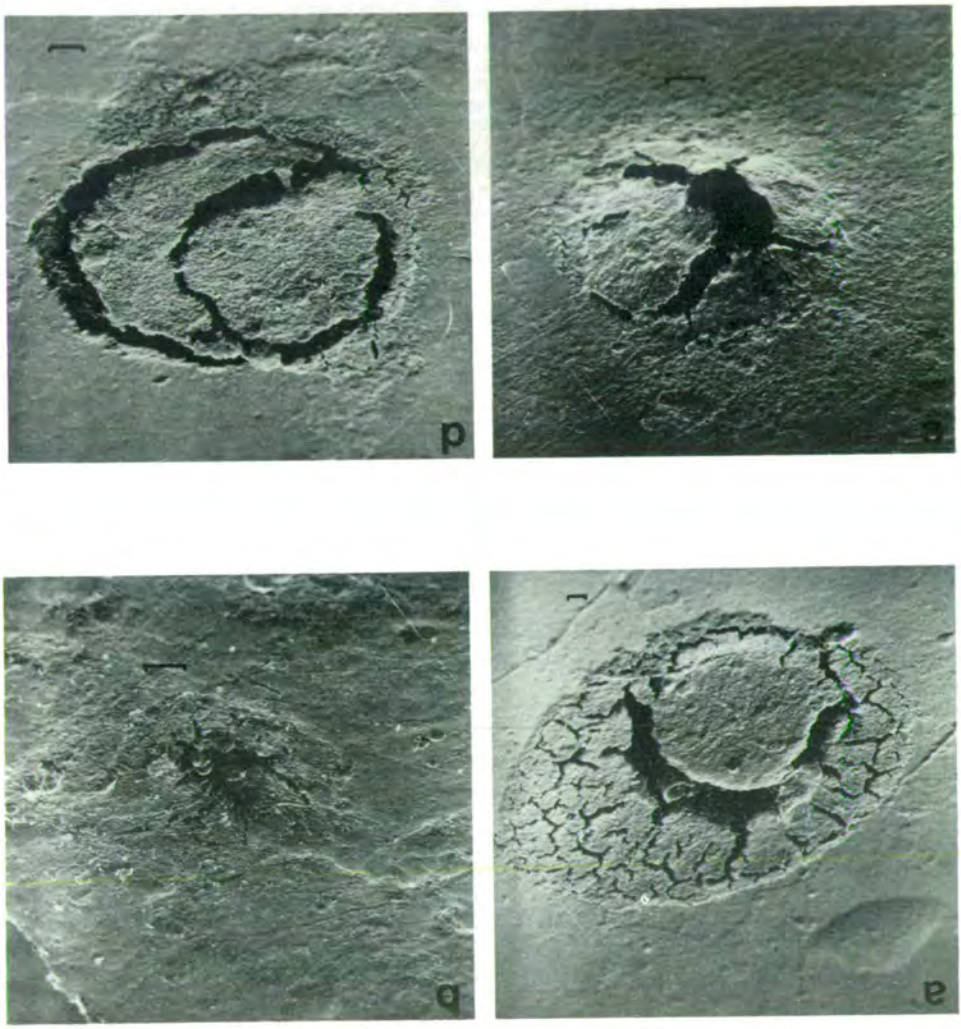


PLATE 25. The surface of the untreated eggshells of tinamous: (a) Elegant Crested Tinamou *Eudromia elegans*; (b) Highland Tinamou *Northocercus bonapartei*; (c) Spotted Tinamou *Nothura maculosa*; (d) Red-winged Tinamou *Rhyncotis rufescens*. A single pore plug is at the centre of each print. All the eggs were obtained from the Western Foundation of Vertebrate Zoology, Los Angeles, U.S.A. Scanning electron micrographs; scale bar = 10 μm in each case.

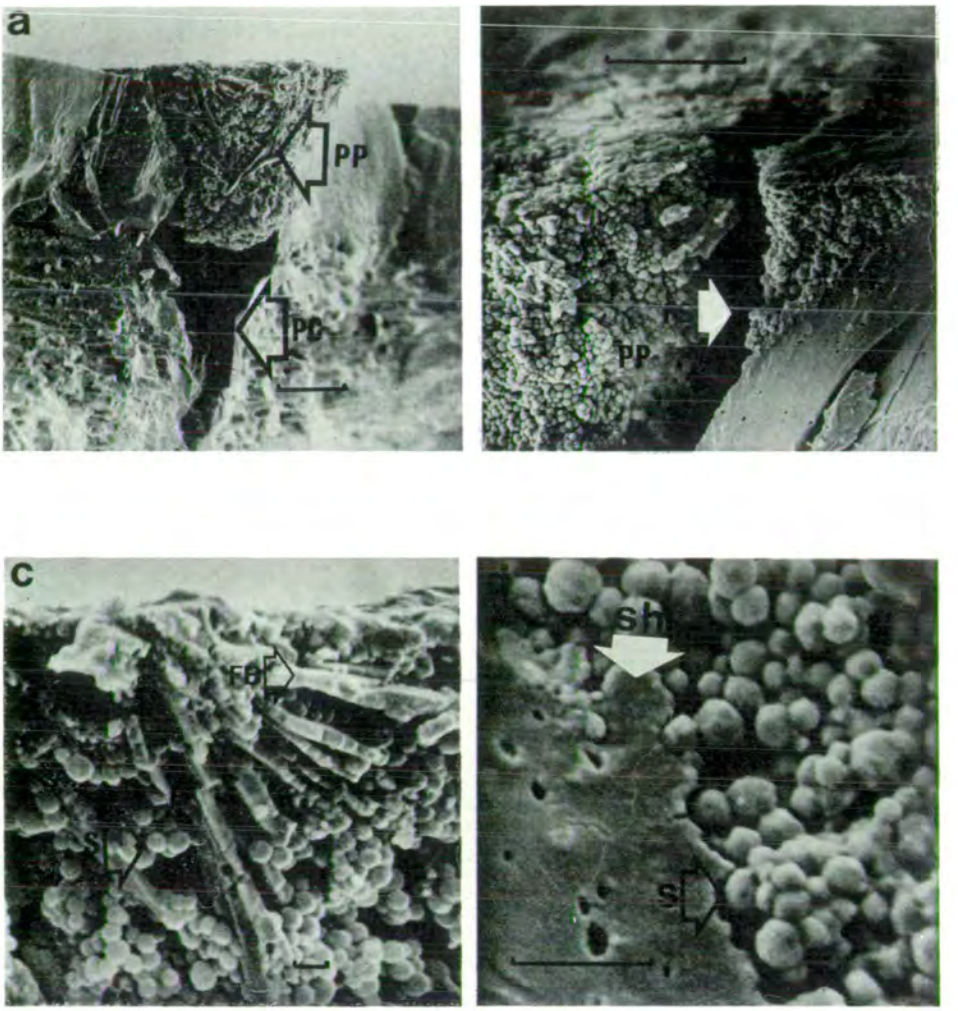


PLATE 26. Details of the plug in the outer orifice of the pore canal in radially fractured eggshell of Elegant Crested Tinamou; origin, Davis, California, U.S.A. (a) Pore plug (PP) in outer orifice of pore canal (PC) (scale bar = 10 μm); (b) fissure (FS) extending through pore plug (PP) (scale bar = 10 μm); (c) details of pore plug showing spheres (S) and fibres (FB) (scale bar = 1 μm), and (d) junction of the calcitic part of the shell (sh) and spheres (s) of the pore plug (scale bar = 1 μm). Scanning electron micrographs.

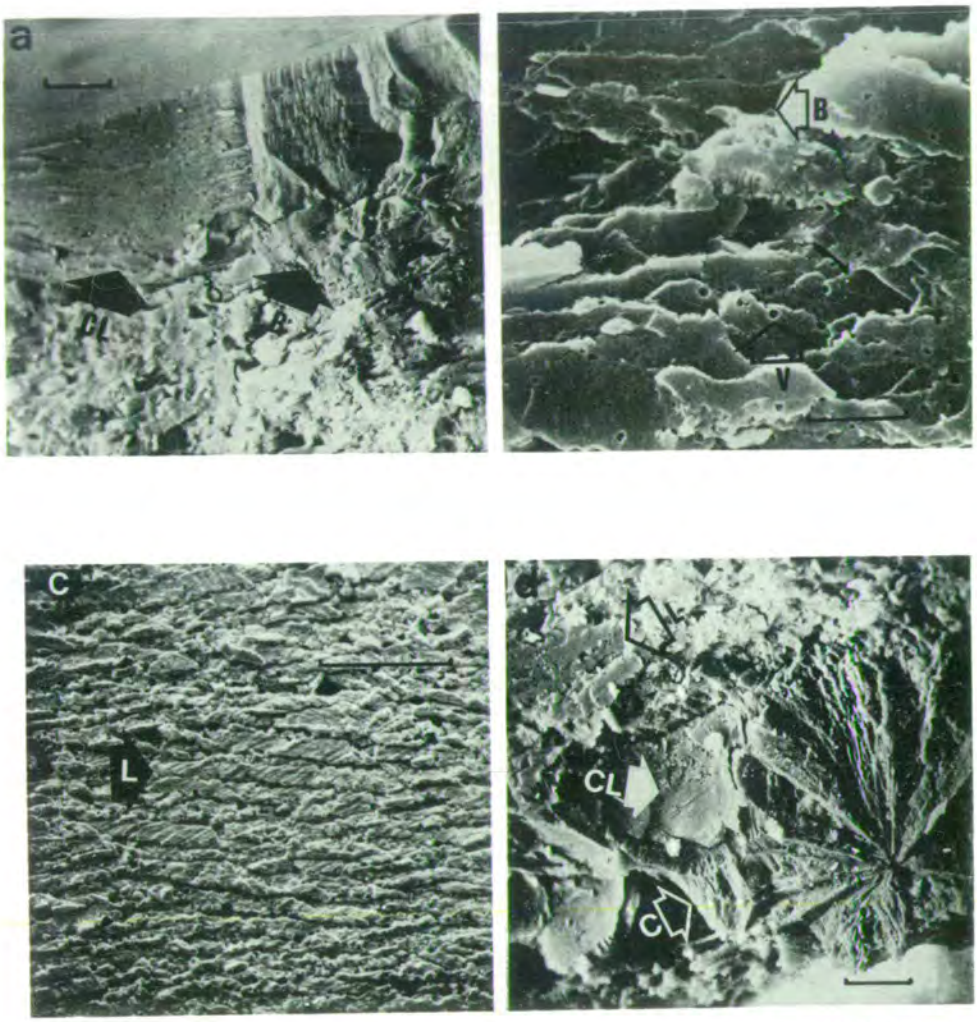
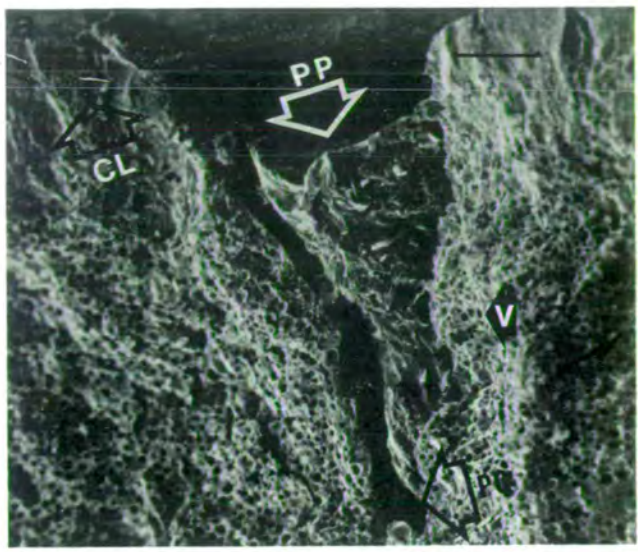
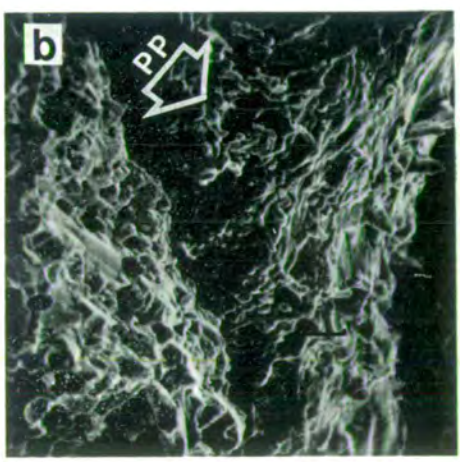


PLATE 27. Details of the shell of Elegant Crested Tinamou as seen in radial section. (a) Outer dense crystalline layer (CL) of the shell overlying blocks (B) of crystalline material (scale bar = 10 μm); (b) details of the blocks (B) of the central stratum of the shell—a few vesicles (V) were present—(scale bar = 10 μm); (c) a polished and acid (carbonic) etched face of a radial section of the shell showing the laminated (L) structure (scale bar = 10 μm), and (d) radial fracture through cone (C) revealing the dense crystalline material (CL) of the cone layer and the abrupt change to the laminated (L) central part of the shell (scale bar = 10 μm). Scanning electron micrographs.

PLATE 28. The surface of the eggshell of Lesser Jaçana *Micropara capensis*; origin, South Africa. PP, plugged pore and PO, pore orifice. Scanning electron micrograph; scale bar = 10 μm.





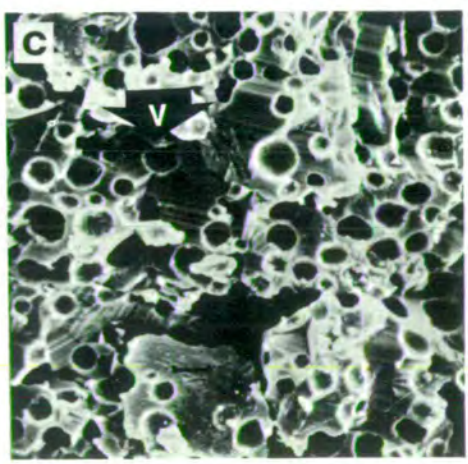


PLATE 29. Ultrastructure of radially fractured eggshell of the Lesser Jaçana; origin South Africa. (a) pore plug (PP) in outer ~~o~~rifice of pore canal (PC)—CL, outer crystalline layer and V, vesicles—scale bar = 10 μm; (b) details of radial surface of pore plug (PP) (scale bar = 1 μm), and (c) vesicles (V) in central stratum of eggshell (scale bar = 1 μm). Scanning electron micrographs.

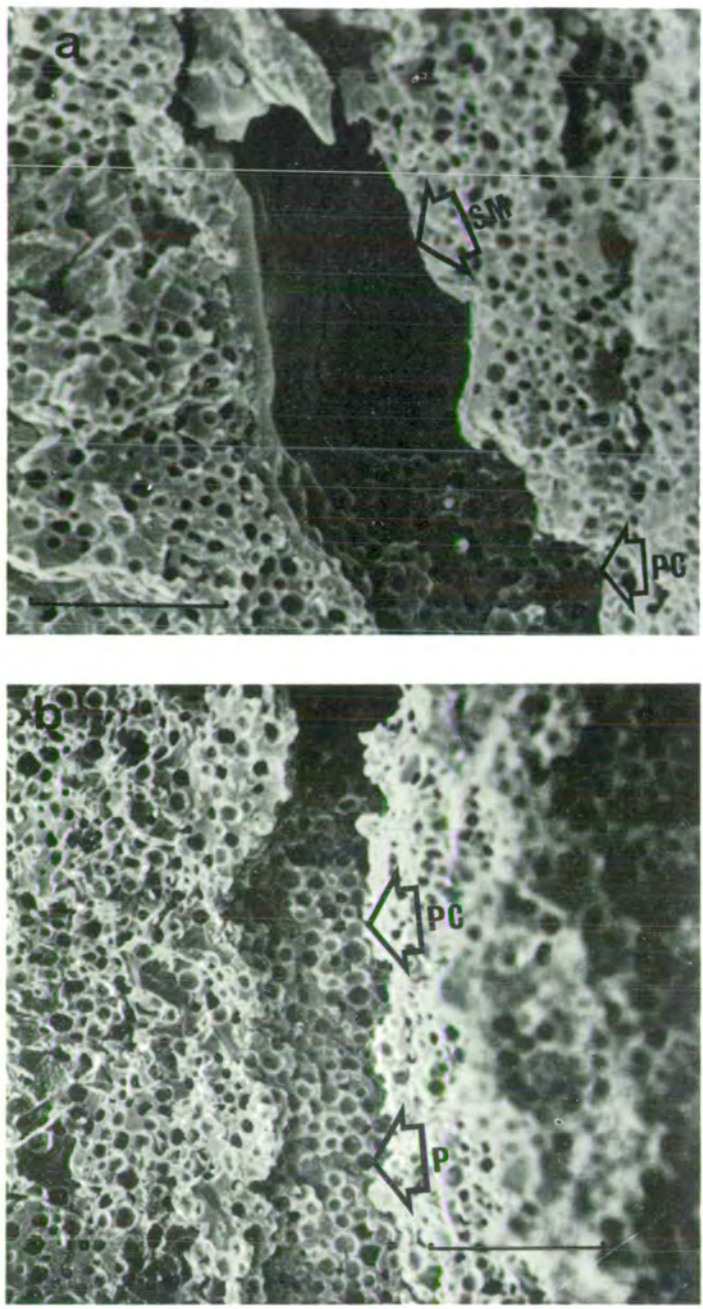


PLATE 30. Details of the pore canal in radial sections of the eggshells of the Lesser Jaçana; origin South Africa. (a) Pore canal (PC) with a lining of smooth material (SM), and (b) the more normal pore canal (PC) with a much pitted (P) wall (scale bar = 10 μm). Scanning electron micrographs.

layer of the shell (Plate 26b). This is evident from Plate 26d where several of the spheres of the plug can be seen to be lodged in shallow depressions in the shell material. This is taken to be evidence that the pore plugs and the outer crystalline material grow concomitantly, at least for a little while, during the final stages of shell formation. Examination with high magnification failed to demonstrate anything other than spheres or short fibres in the plugs, and it was concluded that the irregularly shaped discs of material on the surface of the outermost part of the pore canal shown in Plate 24b were artefacts resulting from treatment with caustic soda. No evidence was obtained in this study to account for the observations of Tyler & Simkiss (1959) that pore canals contained, along their entire length, material stainable (red) with Mallory's triple stain.

In the chemical characterization of the plugs, the electron probe was moved tangentially to the outer edge of pieces of radially fractured shell and the results recorded on charts. As it was not possible to obtain material sufficient for conventional chemical analysis and as the shell could not be polished adequately for quantitative analysis, the characterization had to be based on a comparison between the plug and the adjacent crystalline material. The plug contained trace amounts of calcium and phosphorous, the amount of the latter being less than that in the shell proper. This was taken to be evidence that the spheres were not vaterite, the polymorph of calcium carbonate which occurs as spheres in the cover on the eggshells of the Northern Gannet *Sula bassana* and related species (Tullett *et al.* 1976), as well as on those of the Guira Cuckoo *Guira guira* and Smooth-billed Ani *Crotophagi ani* (Board & Perrott 1979). The spheres in the plug but not the adjacent shell were rich in sulphur. It would appear, therefore, that a material having many disulphide bridges is an important if not the main component of the plugs. Tyler & Simkiss (1959) drew the same conclusion from their histochemical studies.

A laminated arrangement of the shell beneath the funnel-shaped outer orifice in the crystalline layer is a feature of the electron micrographs reproduced in Plates 24b and 26a. The central part of radially fractured shell had the appearance of a dry-stone wall (Plate 27b). To determine whether or not the blocks were rectangles with their long axes in a particular plane, pieces of shell were embedded in plastic so that the orientation of the shell was known. When the plastic had set, it was cut so that radial faces in the longitudinal and latitudinal planes of the shell were obtained. These were ground and then polished with diamond paste (particle size 1·0 μm) and etched with carbonated water. Examination with the SEM (Plate 27c) failed to distinguish between these two faces. Thus it would appear that the central part of the tinamou eggshell is formed from thin, square blocks of calcite (as shown by X-ray diffraction analyses), an arrangement which does not appear to have been noted in the eggshell of other birds examined so far. The organization of the tinamou eggshell gave an unusual appearance to sections ground to a thickness of a few microns and viewed with polarized light. Such preparations revealed the blocks of the central part of the shell and showed that the orientation of the crystals in the outer crystalline layer (Plate 27a) varied from one block to another. They demonstrated, as did scanning electron micrographs, that there was an abrupt change from the inner layer of dense crystalline material (Plate 24a, d) to the middle stratum of the shell. It is evident that more studies need to be done with polarized light microscopy to characterize the crystallography of this middle section of the shell.

THE JAÇANA EGGSHELL

Although the jaçana and tinamou eggshells shared certain features (Fig. 1), they differed markedly in fine structure. Examination with the scanning electron microscope of Lesser Jaçana eggshells showed that the outer orifice of the pore canal was partially occluded with amorphous material situated below the level of the outer surface of the shell (Plate 28). The fissure in this material was partial or complete in the tangential plane of the shell. The pores were freed of the occluding material with boiling sodium hydroxide (aqueous

5% w/v), but the time required (10 minutes) was twice that needed for the tinamou eggshell. This treatment diminished the glossiness of the shell's surface and reduced the intensity of the brown but not the strips of black pigment. As evident from the scanning electron micrographs of pieces of radially fractured shell (Plate 29a), the outer orifice of the pore canal was plugged with material which lacked a discrete structure, there being merely a hint that fibrous material was contained within it (Plate 29b). The plug, which was roughly cone-shaped extended through the outer dense crystalline layer and terminated in that part of the canal situated in the central (major) part of the shell. The latter consisted mainly of calcite (as determined by X-ray diffraction analyses) and contained large numbers of vesicles (Plate 29c). In most cases the fine structure of the wall of the pore canal reflected that of the part of the shell in which it was located (Plate 30a and b). The wall of the occasional pore had a cover of amorphous material in which fissures (Plate 30a) rather than pits (Plate 30b) were a characteristic feature. A probable explanation for this exception was obtained when pieces of radially fractured shell were examined with a light microscope and overhead illumination. The dense crystalline layer on the outer surface of the shell was deeply pigmented (black-brown) whereas most of the central part of the shell was a brilliant white, due no doubt to the vesicles causing total internal reflection of light. Occasionally, however, a strip of pigment (brown-black) was present in the central part of the eggshell. It would seem reasonable to assume that when a pore traversed such a strip, the wall of its canal would be smeared with the pigment thereby giving the fine structure shown in Plate 30a.

The vesicles in the central part of the jaçana eggshell proved an impediment to analysis by the X-ray probe method; they prevented the shell from being polished adequately. Through not having intimate contact with the shell, the plugs were often lost during polishing. Nevertheless, the results obtained with line rather than spot scanning and with photographic rather than chart recording suggest that the plug contains materials rich in iron. As it was estimated that iron was present at a concentration of upwards of 5% of the total, chance contamination of the pore plugs would seem to be an improbable explanation of its occurrence.

DISCUSSION

Board *et al.* (1977) appreciated that their arbitrary classification of the pore systems in avian eggshells should be considered to be tentative because it was based on a small sample (*c.* 60 species of birds). It is not unexpected, therefore, that the present study has revealed a pore system which cannot be accommodated easily in this classification. Although the use of the term 'plugged pore' for the systems found in tinamou and jaçana eggshells implies that a type different from any of those defined by Board *et al.* (1977) has been found, a definition of such a pore might well be construed merely as a description of an accentuated form of the 'occluded', or an abbreviated form of the 'capped' pore system. The lack of fine structure was considered to be a cardinal feature of the material in the orifice of occluded pores (Board *et al.* 1977). As the plug in the pore of the jaçana eggshell did not exhibit a discrete ultrastructure (Plate 29b), it might be inferred that it is an accentuated form of the occluded pore system. Such a conclusion should be deferred, however, until sufficient pore plugs are available for detailed chemical analysis.

By comparable analogy, the pore of the tinamous might be considered derivative of the capped pore system. At the moment, however, such a conclusion is baulked by observations (Plates 26b, d) which suggest that the outer crystalline layer and plug are laid down concomitantly, for a short period at least, during the later stages of shell formation. An abrupt change from the true shell to the cover or cuticle was a common feature of the capped shells studied by Board *et al.* (1977). Tullett *et al.* (1976) considered that the abruptness of the change was evidence of a shift in the secretory products

of the oviduct or the attainment of an inhibitory concentration of some substance in the uterine fluid. For instance, in the formation of the Gannet eggshell, the switch from the deposition of calcium carbonate in the form of calcite to vaterite was attributed to rising phosphate content reaching a concentration inhibitory to the growth of the calcite crystal (Tullett *et al.* 1976). Thus, until more information is available, it would seem acceptable to treat the pore systems of jaçana and tinamou eggshells as distinctive and to apply to them the term, plugged pore.

Board *et al.* (1977) showed that the outer orifice of the pore canals of eggshells of the Rhea *Rhea americana* was partially closed with crystalline material. Further examination confirmed this feature. X-ray probe analyses failed to distinguish between the rough aggregate of crystals in the pore canal and the material in the pore wall. From a morphological standpoint, it might be inferred that the Rhea eggshell provides another example of a plugged pore, distinguished only by the chemical composition of the plug. Alternatively, the pore system in the Rhea eggshell can be interpreted as a very abbreviated form of the reticulate pore systems found in eggshells of Emu *Dromaius novaehallandiae* (Board & Tullett 1975) and Cassowary *Casuarius casuarius* (Board *et al.* 1977). A final decision will have to await an understanding of the mechanisms which cause rough aggregates rather than organized calcite crystals to be formed in the shell gland during the later stages of shell formation.

The adaptive value, if any, of the plugged pores of the tinamou eggshell is not clear. In the discussion of 'problems concerned with eggs', Lack (1968) suggested that waterproofing would be a necessary property of the eggshells of birds such as jaçanas (lily trotters), because they nest in 'wet places'. Studies of the eggs of domesticated birds (Board 1974, Board & Halls 1973a, b) have led to a distinction being made between the water repellency of an eggshell, i.e., the property to shed water when no hydrostatic pressure obtains, and water resistance, i.e., the property of impeding water penetration into the pores when there is a positive hydrostatic pressure. The latter property is conferred on the eggshells of domestic hens, Guinea Fowl *Numidia meleagris* and Aylesbury duck by the cuticle. As no cuticle, or for that matter cover, is present on jaçana eggshells, some other means of providing water resistance has to be sought. The morphology of the pore plug (Plate 29b) is such that it is very tempting to suggest that pressure on its external surface would cause occlusion of the pore orifice. It would, however, require an ingeniously designed experiment to test the water resistance of such a system. Water repellency, on the other hand, may be achieved by the fine structure of the pore canals and the counter-sunk nature of the pore plug. It is likely that, as with water-proofed material (Cassie & Baxter 1944) or the feather of water birds (Rijke 1970), capillary movement of water would be prevented simply because a suitable contact angle between water and the shell's surface around the pore orifice or the wall of the pore canal would not be maintained.

ACKNOWLEDGMENTS

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SUMMARY

By means of scanning electron microscopy and X-ray probe analysis the eggshells of several species of tinamou and jaçana have been investigated. A plugged pore system has been shown which were hitherto unrevealed by other methods. The plug in the pore of the tinamou was rich in sulphur and that of the jaçana eggshell contained upwards of 5% iron.

REFERENCES

BOARD, R. G. 1974. Microstructure, water resistance and water repellency of pigeon eggshells. Br. Poult. Sci. 15: 415–419.

BOARD, R. G. & HALLS, N. A. 1973a. The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. Br. Poult. Sci. 14: 69–97.

BOARD, R. G. & HALLS, N. A. 1973b. Water uptake by eggs of mallard and guinea fowl. Br. Poult. Sci. 14: 311–314.

BOARD, R. G. & PERROTT, H. R. 1979. Vaterite, a constituent of the eggshells of the non-parasitic cuckoos, *Guira guira* and *Crotophagi ani*. Calc. Tissue Intl. (In press).

BOARD, R. G. & TULLETT, S. G. 1975. The pore arrangement in the emu (*Dromaius novaehollandiae*) eggshell as shown by plastic models. J. Microscopy 103: 281–284.

BOARD, R. G., TULLETT, S. G. & PERROTT, H. R. 1977. An arbitrary classification of the pore systems in avian eggshells. J. Zool., Lond. 182: 251–265.

CASSIE, A. B. D. & BAXTER, S. 1944. Wettability of porous surfaces. Trans. Faraday Soc. 40: 546–551.

LACK, D. 1968. Ecological adaptations for breeding in birds. London: Chapman and Hall.

RIJKE, A. M. 1970. Wettability and phylogenetic development of feather structure in water birds. J. Exp. Biol. 52: 469–479.

TULLETT, S. G. 1975. Regulation of avian eggshell porosity. J. Zool., Lond. 177: 339–348.

TULLETT, S. G., BOARD, R. G., LOVE, G., PERROTT, H. R. & SCOTT, V. D. 1976. Vaterite deposition during eggshell formation in the cormorant, gannet and shag, and in 'shell-less' eggs of the domestic fowl. Acta Zool., Stockholm. 57: 79–87.

TULLETT, S. G. & BOARD, R. G. 1977. Determinants of avian eggshell porosity. J. Zool., Lond. 183: 203–211.

TYLER, C. & SIMKISS, K. 1959. A study of the eggshells of ratite birds. Proc. Zool. Soc. Lond. 133: 201–243.

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Vaterite, a Constituent of the Eggshells of the Nonparasitic Cuckoos, *Guira guira* and *Crotophagi ani*

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Summary. The crystalline spherules occurring in patches on the outer surface of the eggshells of *Guira guira* and the bloom on the entire outer surface of those of *Crotophagi ani* were shown by X-ray diffraction analysis to be vaterite.

Key words: Vaterite — Calcium — Eggshells — Cuckoos.

Introduction

Of the three polymorphs of calcium carbonate, calcite, aragonite, and vaterite, the first mentioned is the principal inorganic constituent of avian eggshells [1], and the second is the major component of the calcified shell of reptile eggs [2]. Both polymorphs occur in fossils [3]. The unstable [4] polymorph, vaterite, occurs rarely in sediments [5] or biological materials [6–9]. It has been found, for example, in the reproductive system and egg capsules of the ampullarid snail [9–11] and in the eggshells of the Brown pelican [12]. The latter study did not associate vaterite with a particular part of the shell, but subsequent studies [13] of members of the Pelecaniformes revealed that this polymorph was the major component of a stratum of spherules which overlay the true calcitic shell of the eggs of the Cormorant (*Phalacrocorax carbo*), Shag (*Ph. aristotelis*) and Gannet (*Sula bassana*). Indeed, the fine structure of this outer stratum was sufficiently distinctive for these shells to be assigned to a separate category in an arbitrary classification of eggshells [14]. Vaterite was found also [13] in the mineral deposit on the outer surface of "shell-less" hens' eggs and on a domestic duck egg which had a thin layer of albumen and shell membranes around the outside of a normal shell. From analyses of minerals other than Ca^{2+} in the eggshells of a range of birds, it was deduced [13] that phosphate, a poison of calcite formation [15], was perhaps the cause of the abrupt change in the crystal form in which $CaCO_3$ is laid down on the eggshells of members of the Pelecaniformes. As the size and life style of these sea birds preclude studies of the physiology of their shell gland or the ionic environment of its lumen, the pertinence of the phosphate hypothesis in nature cannot be gauged. Moreover, the conditions leading to the aberrant "shell-less" egg in domestic hens would provide only a part of the story. It is known [16] that the composition of the fluid in the shell gland of domestic hens changes during the period that an eggshell is forming. Thus, for example, the Mg^{2+} concentration increases, a change which is reflected in the eggshell. The concentration of this element rises progressively from a low level in a narrow band immediately behind the inner surface to a peak at the outer surface of the shell [17, 18]. As this feature is not common to the eggshells of all birds [19], there may be important interspecific differences not only in the ionic environment in the shell gland, and changes thereof during shell formation, but also in the means whereby calcite formation continues even though an inhibitor, such as Mg^{2+}, is present.

The appearance of the eggshells of the nonparasitic cuckoos, *Guira guira* and *Crotophagi ani*, has always intrigued egg collectors and curators who have to factor large collections of birds' eggs. The shells of *G. guira* merit the vernacular description, Wedgewood pottery, the blue surface of the shell is overlayed with irregularly shaped white patches. A delicate white bloom covers the shells of *C. ani;* the bloom contains many fine scratches through which the blue color of the underlying shell can be seen. These unusual features attracted our attention because of our interest in the fine struc-

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Table 1. Diffraction patterns of crystals from eggshells compared with synthetic vaterite

Crotophagi ani		Blue-eyed Shag		*Guira guira*		Synthetic vaterite[a] Turnbull (4)		Synthetic vaterite Meyer (36)	
d-spacing A	I/I_1	d-spacing A	I/I_1	d-spacing A	I/I_1	d-spacing A	I/I_1	d-spacing A	I/I_1
4.24	6	—	—	4.24	11	4.23	24	4.23	21
3.57	55	3.57	73	3.56	61	3.57	59	3.57	57
3.29	82	3.30	100	3.28	100	3.29	100	3.30	100
2.73	100	2.73	69	2.72	85	2.73	97	2.73	94
—	—	—	—	—	—	—	—	2.32	6
—	—	—	—	—	—	—	—	2.22	6
—	—	—	—	2.109	7	2.113	7	2.117	15
2.063	47	2.061	58	2.059	52	2.061	66	2.065	62
1.855	12	—	—	1.850	20	1.851	19	1.858	26
1.824	32	1.825	52	1.821	42	1.818	52	1.823	72
1.646	11	—	—	1.647	14	1.643	28	1.647	26

[a] Specimens prepared in the laboratory

ture of avian eggshells in general [13, 14], and our studies have shown that the appearance of the eggshells can be accounted for in terms of an abrupt change from the true (calcitic) shell to an outer cover formed from vaterite. Not only has this study identified birds other than Pelecaniformes that have eggshells made from two of the polymorphs of calcium carbonate but, as both species of nonparasitic cuckoos can be kept in captivity, it has indicated the possibilities for systematic studies of the conditions in vivo which cause this unique switch in crystal form. Moreover, such studies would provide also an opportunity for studying the chemistry of vaterite formation in a warm-blooded animal with the emphasis on a normal rather than on the abnormal situation which results in this polymorph being deposited in gallstones [20, 21], kidney stones [22, 23] and calculi of the bladder [24], etc. It would seem, also, that studies of vaterite formation in warm-blooded animals would provide information which would complement that which to date has been derived from studies of invertebrates [9].

Materials and Methods

Fragments of the eggshells of *Guira guira* and *Crotophagi ani* were obtained by courtesy of Dr. C.J.O. Harrison of the British Museum, London, and those of the blue-eyed shag (*Phalacrocorax atriceps*) were collected (December 1975) in the Antarctic by members of the British Antarctic Survey, Cambridge, England.

SEM Observations

Samples of powders were sprinkled on the surface of double-stick tape mounted on aluminum stubs and coated under vacuum with Au. Pieces of eggshell were glued (DAG 915; Acheson Colloids Ltd., Princes Rock, Plymouth, England) to aluminum stubs and coated under vacuum with Au. A Steroscan S4 (Cambridge Scientific Instruments, Cambridge, England) operating at an accelerating voltage of 10 or 20 kV was used.

X-ray Diffraction

The sample was packed in a glass capillary and examined by the Debye-Scherrer method using copper K_α radiation, at 40 kV and 20 mA. Samples of vaterite prepared by the method of Turnbull [4] were examined in the SEM and analyzed for purity in an X-ray diffractogram.

Results

To the naked eye there was a marked difference between the outer surface of the eggshells of *G. guira* and *C. ani*. The blue surface of the shells of *G. guira* was overlayed with irregularly shaped white patches. A faint white bloom coated the shells of *C. ani*. As boiling with NaOH (5% w/v) did not change the appearance of the eggshells, at least to the unaided eye, it was concluded that the white material was inorganic. This was confirmed by SEM studies and X-ray diffraction analysis of powder scraped from untreated shells. The X-ray diffraction patterns obtained with the Debye-Scherrer camera were essentially identical with those of vaterite prepared in the laboratory (Table 1). X-ray diffractograms of this preparation showed that it contained only a trace of calcite; crystals of the latter were not seen during SEM examination.

The fine structure of the outer surface of the cuckoo eggshells was masked by a film which was removed in boiling NaOH (5% w/v). The origin of the film is not known; it may have been acquired in

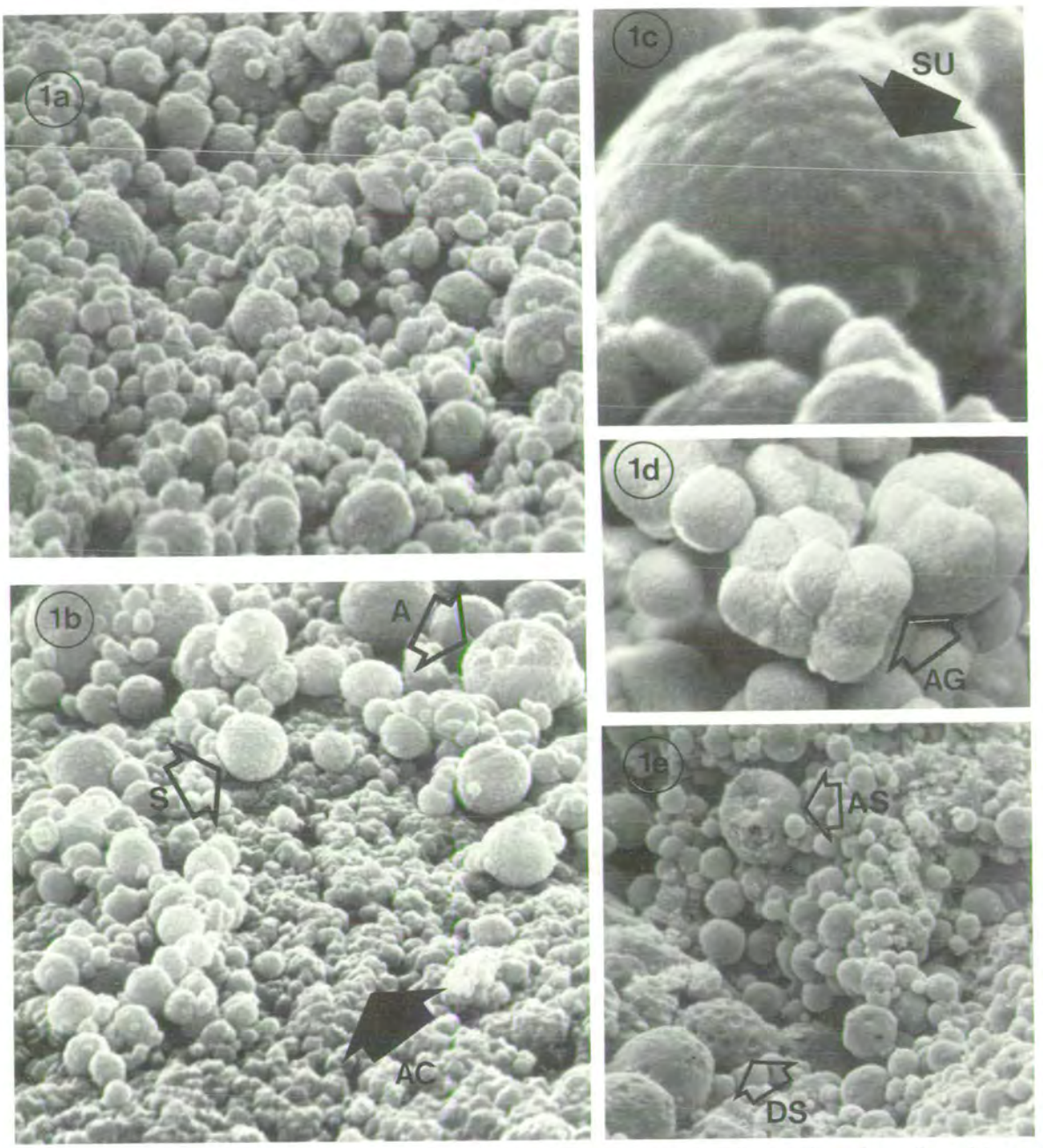

Fig. 1. Electron micrographs of vaterite spherules. **a** A general view of the spherules of a white patch on the surface of the eggshell of *Guira guira*; × 5200. **b** Spherules, both simple (S) and aggregates (A) at the edge of a white patch on the eggshell of *G. guira*. The angular crystals (AC) at the surface of the true shell are evident; × 5300. **c** A spherule from the bloom on the eggshell of *Guira guira* showing spherical units (SU) at the surface; × 21,000. **d** Aggregate spherules (AG) in vaterite prepared by the method of Turnbull [4]; × 5000. **e** Dimpled spherule (DS) and aggregate spherules (AS) in the cover on the eggshell of the blue-eyed shag; × 2400

the nest or during the many years the eggs were stored in a museum. When the film was removed, it was evident that the outer surface of the eggshell of *C. ani* was covered with spheres. Spheres occurred only in the white patches (Fig. 1a) on the outer surface of the eggshells of *G. guira;* the areas between these patches had a rough surface due to the angular form of the surface crystals (Fig. 1b). X-ray diffraction of the material scraped from these areas gave the pattern characteristic of calcite.

As there was no difference in the appearance of the spherules observed in situ on shells cleaned with boiling NaOH and those removed by scraping with a sharp scalpel, it was concluded that the removal of the film had not changed the fine structure of the stratum of spheres overlying the calcitic

shell. It is evident from Fig. 1 a and b that there was a wide range in the size of the spherules and that two or more spherules had fused during formation. A much higher incidence of fused spherules was evident (Fig. 1d) in the vaterite prepared in the laboratory by the method of Turnbull [4]. Indeed, the conglomerates of spherules in this preparation were similar to those found in vaterite recovered from snails [8]. The large dimpled spherules which were a notable feature in the cover on the eggshells of cormorant, shag, and gannet [13] and blue-eyed shag (Fig. 1e) were not seen in the material on the cuckoo eggshells. The large spherules of the latter had a composite appearance when studied at high magnification (Fig. 1c), and in this respect the topology of their surface resembled that of the vaterite prepared in the laboratory. Further evidence that the spherules were polycrystalline came from examination of a spherule which had been fractured when scraped from the eggshell of a cuckoo. It was seen to be formed from fused spheres. In this respect, the spherules on cuckoo eggshells resemble those taken from the reproductive system of ampullarid snails [10].

It was evident from SEM examination of radial sections of cuckoo eggshells that the deposition of calcite in the outer crystalline layer of the shell did not end abruptly; the outer surface was formed from angular crystals of calcite (Fig. 1b; Fig. 2a, c) which gave a rough border between the calcite shell and the stratum of spherules. Not only did the spherules rest on a rough surface, they were present also in the outer part of the pore canal (Fig. 2b, d). In the latter situation there was a suggestion that spherules may well have been forming at the same time as the calcite. Thus some spherules appear to be embedded in the wall of the pore canal (Fig. 2d).

Discussion

The present study of the eggshells of two species of nonparasitic cuckoos, *Guira guira* and *Crotophagi ani*, has shown that a switch from the deposition of calcite to that of vaterite during formation of an eggshell occurs in birds other than members of the Pelecaniformes [13]. It adds, moreover, to the rather restricted list of biological materials in which vaterite is laid down naturally [6–9] rather than as a response to damage, as in mollusc shells [11], or in pathological conditions such as gallstones [20, 21], kidney and related calculi [22–24], and aberrant otoliths of cod [25]. This study raises two questions: Why is vaterite formed? How is vaterite formed?

To account for vaterite on the eggshells of gannet and related species, Tullett et al. [13] invoked an argument about the need for some sea birds (e.g., the cormorant) to avoid large increases in the amount of storage calcium in the bones during the breeding season so that there was not a drain on the energy required for flight. Likewise, the structural strength of the skeleton of the gannet, birds which plunge from upwards of 50 feet when catching their prey in the sea, was put forward as a possible reason for these birds having vaterite in their eggshells—the specially adapted bones being unable to accommodate sufficient reserves of Ca^{2+} for shell formation. In both examples, it was deduced [13] from analyses of the phosphate content of the shell that release of this substance during the breakdown of skeletal rather than medullary bone would lead to levels in the lumen inhibitory to calcite formation. These arguments as to the cause of vaterite formation would seem to be inapplicable to the eggs of the nonparasitic cuckoos and, if there is a *raison d'être* for the occurrence of vaterite, some selective pressure outwith the basic physiology of the parent has to be sought. Although it is not yet possible to identify such a pressure, the nesting habits of the nonparasitic cuckoos might be expected to pose problems to the egg. Thus the naturalists' description of nests containing upwards of 30 eggs intermingled with vegetation might be construed as evidence that the shell requires protection from mechanical damage due to collisions with other eggs and from microbial colonization of its outer surface. It has been demonstrated [26] that an eggshell covered with vaterite spherules is remarkably resilient to damage from repeated assault with a ball bearing, but the resilience is lost when the cover is removed. Although the author did not speculate as to the nature of the defense offered by the cover, it would seem reasonable to assume that the vaterite spherules collapsed under impact thereby protecting the underlying calcite. It has been noted [27] also that microorganisms digest the organic cuticle when they grow on the surface of the hens' eggshell. As digestion frees the pores of capping material, the contents of the eggs are rendered liable to microbial infection. It may be therefore that selection has favored a shell with an inorganic cover on which microbial colonization, leading to a weakening of the physical defenses of the egg, cannot occur. The surmise [13] that the poisoning of calcite formation by phosphate leads to an abrupt switch from calcite to vaterite formation on the eggshells of members of the Pelecaniformes does not take into account all the factors which can influence the crystal form of calcium carbonate. Now that two species of birds offering the prospect of laboratory studies are available, future studies can be put into a wider perspective.

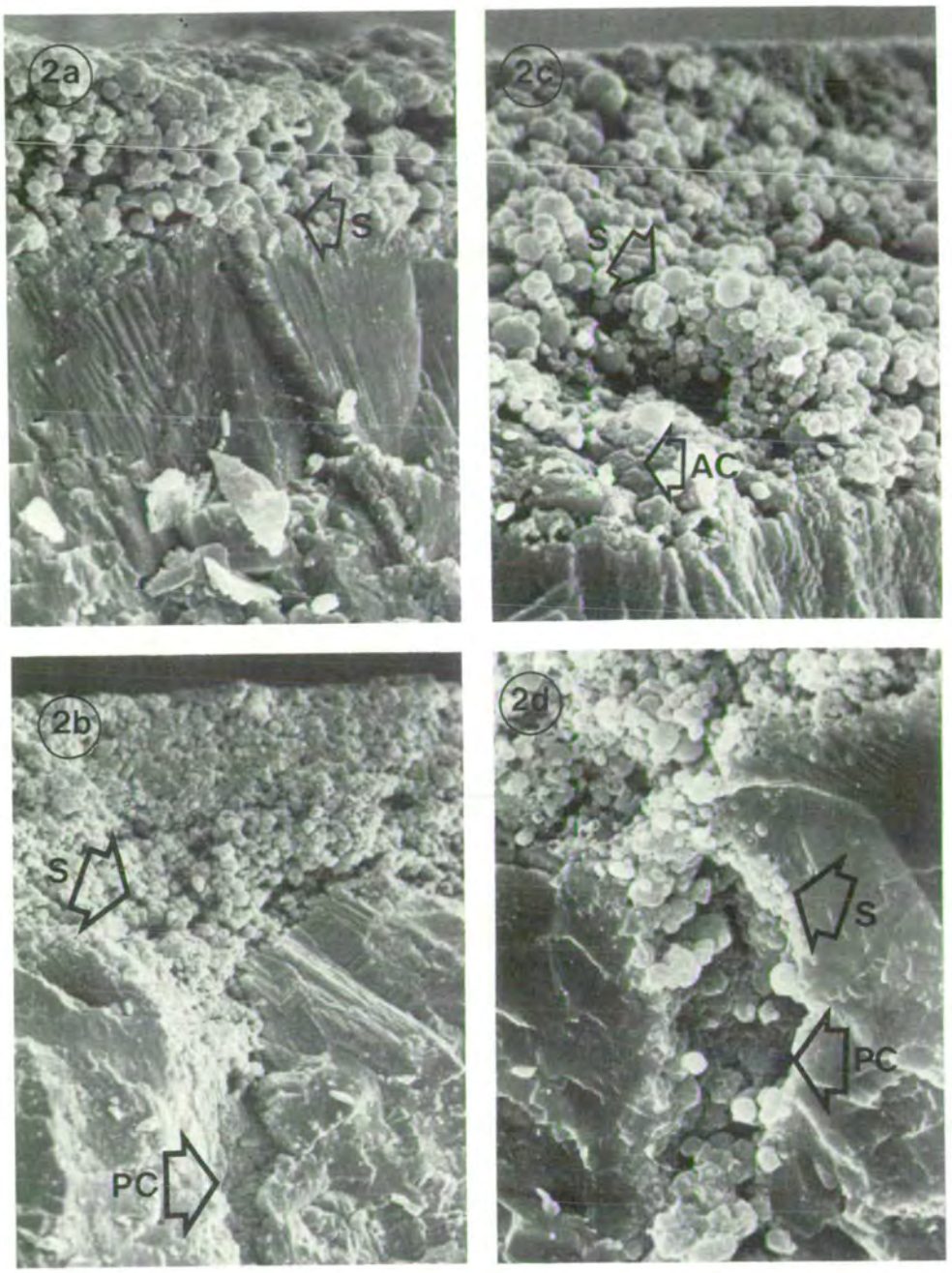

Fig. 2. Electron micrographs of the outer edge of radial sections of the eggshell of *Guira guira* and *Crotophagi ani*. **a** Spherules (S) on the outer surface of the eggshell of *C. ani*; × 2000. **b** A stratum of spherules (S) overlying the outer orifice of a pore canal (PC) in the eggshell of *C. ani*; × 1150. **c** Spherules (S) overlying angular crystals (AC) on the outer surface of the eggshell of *G. guira*; × 2400. **d** Spherules (S) embedded in the wall of the pore canal (PC) in the eggshell of *G. guira*; × 2000

Although vaterite can now be regarded as a naturally albeit rarely occurring form of calcium carbonate in biological materials, little is known about the conditions leading to its deposition or, for that matter, to the maintenance of this metastable polymorph [28] once it is formed. Unlike calcite and aragonite, the vaterite crystal is disordered [29]. It has been suggested [30] that its hexagonal symmetry with a pseudo cell [31] would cause stacking problems along the c-axis. There is ample evidence of its instability when in contact with water [4, 32]. The properties of vaterite have been determined on material obtained by either rapid precipitation [4] or slow ionic diffusion [30]. With rapid separation from the mother liquor and drying, the former method gives mainly vaterite whereas ionic diffusion gives a mixture of all three polymorphs. It has been demonstrated repeatedly that cations such as Ba^{+} and Mg^{2+} tend to increase the proportion of vaterite [33, 34]. When examining factors that might determine which of the polymorphs would form in sea water, Simkiss [35] noted that Mg^{2+} favored the formation

of aragonite and a low pCO_2 of vaterite during the relatively slow precipitation of crystals in synthetic sea water. In biological systems the influence of the ionic environment, pCO_2, temperature, etc. may well be muted or accentuated by nucleation sites provided by organic material. Indeed, this has been shown by Kitano [34], and in the review by Watabe [8] of crystal growth of calcium compounds and biological systems, emphasis was given to the important role of a fibrous organic matrix in the crystallization process in general. Along with collaborators, this worker demonstrated [9] a matrix formed of proteins and acid mucopolysaccharides in the vaterite-rich spherules in *Pomacea palludosa*. Thus future studies of the conditions leading to vaterite formation in the lumen of the oviduct of *G. guira* and *C. ani* would have to give at the outset equal weighting to the ionic environment of the uterine secretion and its content of fibers having the potential to provide nucleation sites.

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References

1. Wilbur, K.M., Simkiss, K.: Calcified shells. M. Florkin and E.H. Stotz (eds.): In Comprehensive Biochemistry. Elsevier Publishing Co., Amsterdam, 1968
2. Erben, H.K.: Ultrastrukturen und Mineralisation rezenter und Fossiler Eischalen bei Vogeln und Reptilien. Biamineralization Forschingsberite, Vol. 1, pp. 1–66. F.K. Schattaner, Stuttgart, New York, 1970
3. Hall, A., Kennedy, W.F.: Aragonite in fossils, Proc. R. Soc. Biol. **168**:377–412, 1967
4. Turnbull, A.G.: A thermochemical study of vaterite, Geochim Cosmochim. Acta **37**:1593–1601, 1973
5. Bentor, Y.K., Gross, S., Heller, L.: Some unusual minerals from the mottled zone complex, Israel, Am. Mineral. **48**:924–930, 1963
6. Hall, A., Taylor, J.D.: The occurrence of vaterite in gastropod eggshells, Mineral. Mag. **38**:521–522, 1971
7. Lowenstam, H.L., Abbott, D.P.: Vaterite: a mineralization product of the hard tissues of a marine organism (Ascidiacea), Science **84**:363–365, 1975
8. Watabe, N.: Crystal growth of calcium carbonate in biological systems, J. Cryst. Growth **24/25**:116–122, 1974
9. Watabe, N., Meenakshi, V.R., Blackwelder, P.L., Kurtz, E.M., Dunkelberger, D.G.: Calcareous spherules in the gastropod, *Pomacea palludosa*, Belle W. Baruch. Libr. Mar. Sci. **5**:283–308, 1976
10. Meenakshi, V.R., Blackwelder, P.L., Watabe, W.: Studies on the formation of calcified egg-capsules of ampullarid snails. I. Vaterite crystals in the reproductive system and the egg-capsules of *Pomacea palludosa*, Calcif. Tissue Res. **16**:283–291, 1974
11. Meenakshi, V.R., Blackwelder, P.L., Hare, P.E., Wilbur, K.M., Watabe, N.: Studies on shell regeneration—I. Matrix and mineral composition of the normal and regenerated shell of *Pomacea palludosa*, Comp. Biochem. Physiol. **50A**:347–351, 1975
12. Gould, R.W.: Brown pelican eggshells: X-ray diffraction studies, Bull. Environ. Contam. Toxicol. **8**:84–88, 1972
13. Tullett, S.G., Board, R.G., Love, G., Perrott, H.R., Scott, V.D.: Vaterite deposition during eggshell formation in cormorant, gannet and shag and in "shell-less" eggs of the domestic fowl, Acta Zool. (Stockh.) **57**:79–87, 1976
14. Board, R.G., Tullett, S.G., Perrott, H.R.: An arbitrary classification of the pore systems in avian eggshells, J. Zool. (Lond.) **182**:251–265, 1977
15. Simkiss, K.: Phosphates as crystal poisons of calcification, Biol. Rev. **39**:487–505, 1964
16. El Jack, M.H., Lake, P.E.: The content of the principal inorganic ions and carbon dioxide in uterine fluids of the domestic hen, J. Reprod. Fertil. **13**:127–132, 1967
17. Brooks, J., Hales, H.P.: Strength of the shell of the hens' egg, Nature **175**:848–849, 1955
18. Itoh, H., Hitano, T.: Variation in magnesium and phosphorous deposition rates during eggshell formation, Poult. Sci. **43**:77–80, 1964
19. Board, R.G., Love, G.: A survey of Mg^{++} content in avian eggshells, Comp. Biochem. Physiol. (submitted)
20. Bogren, H., Larrson, K.: Crystalline components of biliary calculi, Scand. J. Clin. Lab. Invest. **15**:457–462, 1963
21. Sutor, J.D., Wooley, S.E.: Gallstones of unusual composition: calcite, aragonite and vaterite, Science, **159**:103–114, 1968
22. Gruenberg, W.: Karbonat-Harsteine herbivores Saügetiere, Zentralbl. Veterinärmed. [A] **18**:767–823, 1971
23. Sutor, J.D., Wooley, S.E.: Animal calculi: X-ray diffraction study of their crystalline composition, Res. Vet. Sci. **11**:299–301, 1970
24. Sutor, J.D., Wooley, S.E., Jackson, O.F.: Crystalline material from the feline bladder, Res. Vet. Sci. **11**:298–299, 1970
25. Palmork, K.H., Taylor, M.E.U., Coates, R.: The crystal structure of aberrant otoliths, Acta Chem. Scand. **17**:1457–1458, 1963
26. Tyler, C.: The snapping strength of the eggshells of various orders of birds, J. Zool. (Lond.) **159**:65–77, 1969
27. Board, R.G., Loseby, S., Miles, V.R.: Microbial growth on the cuticle on the hens' eggshell, Br. Poult. Sci. (submitted)
28. Rock, P.A., Gordon, A.Z.: Determination of Gibbs energies for solid-solid phase transitions in electrochemical double cells without liquid function: calcite, aragonite and vaterite, J. Am. Chem. Soc. **98**:2364–2365, 1976
29. Jenkins, H.B.D., Pratt, K.F., Smith, B.T., Waddington, T.C.: Lattice potential energies for calcite, aragonite and vaterite, J. Inorgan. Nucl. Chem. **38**:371–377, 1976
30. Kamhi, S.R.: On the structure of vaterite, $CaCO_3$, Acta Cryst. **16**:770–772, 1963
31. Morgan, P.E.D., Carroll, P.J., Lange, F.F.: Crystal structure of $YSiO_2N$ and a reappraisal of the 'vaterite' type, YBO_3, Mar. Res. Bull. **12**:251–260, 1977
32. Bischoff, J.L.: Catalysis, inhibition and the calcite aragonite problem. II. The vaterite-aragonite transformation, Am. J. Sci. **266**:80–90, 1968
33. Kitano, Y.: The behaviour of various inorganic ions in the separation of calcium carbonate from a bicarbonate solution,

Bull. Chem. Soc. Jpn. **35:**1973–1980, 1962
34. Kitano, Y.: On factors influencing the polymorphic crystallisation of calcium carbonate found in marine biological systems. Rec. Res. Hydrosphere, Atmosphere and Nuclear Biochemistry, pp. 305–319, 1964
35. Simkiss, K.: Variations in the crystalline form of calcium carbonate precipitated from artificial sea water, Nature **201:**492–493, 1964
36. Meyer, H.J.: Struktur and Fehlordnung des Vaterits, Z. Krist. **128:**183–212, 1969

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The phosphate-rich cover on the eggshells of Grebes (Podicipitiformes)

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Spheres of a nut-like morphology - a "kernel" formed from spherules contained in a "shell" of globular sub-units - were a unique feature of the cover on the eggshells of six species of the order Podicipitiformes: Red necked Grebe (Podiceps grisegena), Great Crested Grebe (Podiceps cristatus), Little Grebe (Tachybaptus ruficollis), Black-necked Grebe (Podiceps nigricollis), Slavonian Grebe (Podiceps auritus) and Pied-billed Grebe (Podilymbus podiceps). The spheres did not give a pattern with X-ray diffraction; the innermost ones were embedded in the dense crystalline layer on the outer surface of the calcitic portion of the shell. Electron-probe microanalysis revealed that the cover of spheres contained principally oxygen, calcium, phosphorus and sulphur and infra-red analysis demonstrated the presence of phosphate. The outer surface of the stratum of spheres was bounded by a thin, fissured layer of amorphous material.

These observations raise questions about the ionic environment obtaining in the shell gland towards the end of shell formation and the adaptive significance of the cover on the eggshells of members of the Podicipitiformes.

Contents

Introduction

The postulate (Lack, 1968; Board, 1982) that the shells of birds' eggs laid in wet places need to be waterproofed so that flooding of the pore canals does not asphyxiate the embryo has prompted extensive surveys of the pore systems in such eggshells. These have revealed that the outer orifice of pores and, in general, the entire outer surface of eggshells laid in wet, muddy or exposed nests are covered with a layer of spheres (Board, 1980; Board & Scott, 1980; Board, Tullett & Perrott, 1977). Vaterite (Tullett et al., 1976) is a major component of the spheres on the eggshells of shags (order Pelecanidae) and two species of non-parasitic cuckoo, Crotophagi ani and Guira guira (Board & Perrott, 1979a) whereas amorphous calcium phosphate occurs in the covering layer on the eggshells of flamingoes (Board, 1981a) and megapodes (Board et al., 1982). A poorly characterized glycoprotein is the main component of the spheres on the outer surface of the eggshells of the domestic hen (Wedral et al., 1974). The African Lily Trotter (Microparra capensis) and Tinamous provide exceptions (Board & Perrott, 1979b) in that the outer orifice of the pores contains a loose fitting plug of amorphous material, probably organic in nature, but the shell is uncovered.

Not only have these studies characterised the morphological features of eggs laid in inimical environments, they have indicated also that the physiology of birds, especially with respect to their shell glands, have been modified such that eggshells are adapted to the nest cup environment. In the context of an inimical environment, Grebe eggshells are obvious candidates for study because they are incubated on floating platforms of sodden vegetation and become heavily stained during incubation. Indeed Lack (1969) surmised that Grebe eggshells were waterproofed by the chalky cover, a characteristic feature of freshly laid eggs. The outer surface of the cover has been described as leather-like (Nathusius, 1882, 1887; Schönwetter, 1962) and it was identified with the cuticle by Tyler (1969) who referred to

the underlying material as the chalky cover. The last mentioned author also referred generally to studies with polarized light and stated that "the cover consists of organic matter in which are embedded crystals of calcite". Schmidt (1963) noted the occurrence of sphaerites in the outer edge of the true shell as well as in the cover of the sections of the eggshells of Podiceps cristatus and Podiceps minor examined with polarized light. It is evident from these few studies of Grebe eggshells that the chemical composition and fine structure of the cover is poorly defined.

In this communication we give a detailed description of the fine structure of the cover on the eggshells of six species of Grebe, show that amorphous calcium phosphate is a major component and reconsider the use of the terms cover and cuticle in the context of shell gland physiology.

Materials & Methods

Source of eggs

Eggshells of the following six species of the order Podicipitiformes were studied: (1) the Black-necked Grebe, Podiceps nigricollis - sometimes referred to as the Eared grebe, Podiceps caspicas; (2) the Little Grebe, Tachybaptus ruficollis, (3) the Slavonian Grebe, Podiceps auritus; (4) the Red-necked Grebe, Podiceps grisegena; (5) the Great Crested Grebe, Podiceps cristatus, and (6) the Pied-billed Grebe, Podilymbus podiceps. Eggshells of 1 (referred to as the Eared Grebe) and 3, both of which had been recently collected in the U.S.A., were gifts from Paul Sotherland and Ralph Ackerman respectively; those of 2, 4 and 5 and some of 1 were taken from the eggshell collection which Professor C. Tyler kindly donated to one of the

authors (RGB); and those of 6 were collected from nests at the Wildfowl Trust, Slimbridge, Glos or adjoining gravel pits. With the last mentioned, 4 eggs were collected before incubation began; another two eggs from the same sites contained embryos with down-covered bodies. The Greater Flamingo (Pheonicopterus ruber roseus) eggshells were gifts from Professor G.V.T. Matthews (The Wildfowl Trust, Slimbridge, Glos); the Malee fowl eggshells (Leipoa ocellata) were obtained from Dr. Roger Seymour, University of Adelaide, Australia and those of the Gannet (Sula bassana) were collected by RGB from the Bass Rock, North Berwick, Scotland. The eggs of domestic hens were collected from a local farm. The nomenclature used above was taken from Cramp & Simmons (1977).

Scanning Electron Microscopy

A Cambridge "Stereoscan" S4 was used for the majority of observations of untreated fragments of eggshells or pieces which had been boiled for 5 minutes in water, a domestic detergent (Teepol), or 5% (w/v) aqueous solution of sodium hydroxide. In other examinations, radial sections of untreated eggshells were mounted in plastic (Pasticraft, Turner Research Ltd., Leeds, England), polished on diamond paste down to 1µm grade and etched with carbonated water. The eggshell samples were glued (DAG 915, Acheson Colloids Ltd., Plymouth, England) onto aluminium stubs and then sputter-coated with a thin conducting layer of gold. Specimens were stored in a desiccator while awaiting examination. A JEOL-35c was used to study the ultrastructure of the speres in the outer cover on the eggshells.

Electron-Probe Microanalysis

This was performed on the polished sections (gold coated) and also on untreated fragments of uncoated eggshells. A JEOL JXA-50A instrument

(JEOL (UK) Ltd.), fitted with crystal spectrometers and an ECON energy-dispersive analyser (EDAX (UK) Ltd.) were used in the initial experiments and a JEOL Superprobe 737 with computer control in the final stages of the study.

X-ray diffraction

The sample was packed in a glass capillary and examined by the Debye-Scherrer method using copper Kα radiation with a nickel filter to remove the CuK β line. The diffraction patterns were compared with those obtained with pure samples of calcite, vaterite and aragonite.

Infra-red Analysis

Powdered cover in a Nujol (a mineral oil) mull (paste) was investigated with infra-red radiation, using a Perkin Ellmer 197 spectrophotometer for calcium phosphate.

Results

Structure

Three features (Fig 1a, b) common to the eggshells of all six species of Grebe included in this study will be discussed: (1) the cover on the outer surface of the shell; (2) the transition zone in which spheres are embedded in dense crystalline material of the true shell, and (3) the pores and large

basal caps on the apices of the cones. Although eggshells of all six species of Grebe were studied in detail, only illustrations of the Great Crested Grebe are presented because the eggs were examined immediately following collection from local nests.

The Cover

The shells of freshly laid eggs of the Great Crested Grebe had an off-white, matt surface and were smeared in places with mud. Incubated eggs were stained brown and polished, mud only occurring at the poles. The eggshells of the Black-necked and Pied-billed Grebes, both of which had been collected shortly after oviposition, resembled those of the Great-crested Grebe. The eggshells of the Black-necked, Slavonian, Little and Red-necked Grebe taken from the Tyler collection were polished and stained a mahogany colour. Inspection of whole clutches of Grebes eggs in the Tomkinson Collection at the Wildfowl Trust, Slimbridge, Glos, supported the observations (Cramp & Simmons, 1977) that the off-white matt finish is characteristic of eggs collected soon after oviposition and that incubation causes polishing and staining.

Examination with the Scanning Electron Microscope (SEM) showed [Plate I(a)(d)] that the outer surface of recently laid as well as incubated eggs of all six species of Grebe was covered by amorphous material containing a net work of cracks, approximately 1-10μm wide. These cracks were evident also in casts (Board, 1981b) of the outer surface of whole eggs. The outer-layer of amorphous material was unaffected by boiling for 5 minutes in water or Teepol but it was thinned and fissures removed by boiling sodium hydroxide (5% w/v aqueous). The spheres underlying the outer layer of unincubated eggs lacked fine structure [Plate II (c)] whereas those of incubated ones appeared to have been smeared with amorphous material [Plate II(d)].

The outer layer of amorphous material and underlying spheres [Plate II(a)] were unaltered by boiling in water or Teepol for 5 minutes. A boiling solution of sodium hydroxide removed the outer amorphous layer and revealed [Plate II(b)] the nut-like morphology - a "shell" of globular subunits surrounding a "kernel" of spherules [Plate III(c)(d)] - of the spheres composing the cover. The nut-like morphology was evident also [Plate III(a)(b) in spheres trapped in the outer part of the calcitic part of the shell especially when the latter were cooled with liquid nitrogen before fracture. As Pooley (1979) has shown that an organic coat occurs on the spheres in the eggshells of the White Pelican, Pelecanus erythroxhyncus, we are of the opinion that the hydroxide treatment freed the spheres on Grebe eggshells of an organic coat thereby rendering them fragile.

The Transition zone

Spheres - identified with the sphaerites described by Schmidt (1963) - were evident in the outer part of the polished radial sections of Grebe eggshells examined with polarized light. When the specimen was rotated, such that the columnar crystals traversing the radial section of shell were extinguished in turn, it was evident that the columns began at the point of fusion of the cones and terminated in a feathered edge beneath the outer layer of spheres. This evidence together with that obtained from studies with the SEM led us to conclude that there is a gradual transition from the calcitic shell to the cover in the Grebe eggshells. The radial width of the transition zone appeared to vary in accordance with egg size. Thus it was most extensive in the heaviest eggs (eg. those of the Great Crested Grebe; average egg weight 42g) and least in the Little Grebe eggshells (average weight, 14g; Cramp & Simmons, 1977).

The Pores and Cone Layer

The pore canal originated between the cones [Plate IV(b)] on the inner surface of the shell, traversed the shell radially [Plate I(b)] and terminated at the outer part of the transition layer. As the outer pore orifice was located at the bottom of an interfacial undulation, it was overlayed with the maximum thickness of cover [Plate I(b)]; the outermost portion of the pore canal also contained a crudely fitting plug of spheres [Plate II(b)]. The morphology of the inner orifice of the pore canal differed markedly from those described elsewhere. Whereas the pore canal normally begins at the junction of conical projections having grooved apices, those of all of the Grebe's eggshell included in this study appeared in radial section [Plate IV(c)] to originate between cones that had no contact with the shell membranes. Indeed, examination of radial sections at low magnification gave the impression that there was a continuous gap between the inner face of the shell and the shell membranes, due to the unusual morphology of the cone layer. In the unincubated eggs of the Great Crested Grebe, the grooved, flat basal caps were joined by thin necks to bulbous "cones" that fused to form the base of the palisade layer [Plate IV(c)(d)]. The shell membranes could not be pulled away from shells of unincubated eggs and Plate IV(b) was taken from a shell with the membranes removed by boiling for 5 minutes in sodium hydroxide (5% w/v aqueous). The membranes were easily detached from the shells of incubated eggs and the basal caps were fractured tangentially [Plate V(a)(c)]. The fractured face of the basal caps revealed a central vacuole, probably bounded by a membrane, surrounded by smaller vacuoles and crystals having a radial orientation [Plate V(b)] in the tangential plane. In radial sections, the large vacuole was located at the junction of the eisospherite and exospherite of Schmidt, the crystals of the exospherite having a marked radial symmetry. The surface of the cones showed signs of chemical attack due to the embryo's sequestration of calcium [Plate V(d)].

Chemical composition

Although the spheres in the cover did not dissolve in boiling sodium hydroxide, an observation in accord with that of Tyler (1969), we did not see effervescence when the cover was scraped into mineral acid, as he had done. When plastic embedded shell with a finely polished radial face was immersed in water through which carbon dioxide had been bubbled, the spheres in the cover dissolved leaving a honey combe of plastic [Plate IV(a)]. The transition zone dissolved more rapidly than the bulk of the calcitic layer presumably as a consequence of the greater surface area of the sphere-rich zone. These observations with the SEM permit a modification of the interpretation made by Tyler (1969) who studied acid etched shells with a light microscope: "the eggshell of Podicipitiformes give with acid etching an outer layer, about 10-15%, which is unetched and the rest divides into two layers, namely a middle layer which etches rather more rapidly than the innermost layer". There appears to be a contradiction in our failure to demonstrate gas evolution from cover treated with hydrochloric acid but to cause rapid dissolution of spheres by simply immersing them in carbonated water. It is our opinion that an outer cover of organic material, as has been demonstrated by Pooley (1979) - see above - protects the underlying mineral portion of the spheres. When this was damaged by polishing, carbonated water reacted with the underlying minerals.

The cover gave no X-ray diffraction pattern whereas the underlying shell gave the characteristic pattern of calcite. Infra-red analysis of powdered cover gave a pattern for calcium phosphate.

Electron probe analysis with the JEOL Superprobe 737 showed that the cover on Grebe eggshells contained oxygen, sulphur, calcium and phosphorus. Indeed the peak concentration of phosphorus and sulphur occurred in the cover (Fig. 1). This was the case also with the eggshells of the Andean Flamingo

and Mallee fowl, the covers on which contain amorphous calcium phosphate (Board 1981a; Board et al. 1982) as well as in the vaterite-rich cover (Tullett et al. 1976) on Gannet eggshells (Fig. 1). With the domestic hen, the concentration of phosphorus but not sulphur increased in the outer part of the calcitic shell. It is known (Nathusius, 1882, 1887; Schönwetter, 1960; Tyler, 1969) that the outer surface of Grebe eggshells is bounded by a thin layer of organic material; the electron probe analysis did not demonstrate an exceptionally large amount of sulphur in this material.

Discussion

This study has identified two topics for discussion: (a) the adaptive significance of the fine structure of Grebe eggshells, and (b) the physiology of these birds at the time of shell formation.

Adaptive significance

Lack's (1968) contention that eggs laid in wet places need to be waterproofed - presumably to prevent asphyxiation of the embryo - has been supported by the demonstration (Board & Halls, 1973) that the cuticle, a layer of glycoprotein spheres, on the hens' eggshell prevents water from entering the pore canals unless hydraulic pressures obtain. The observations that eggshells laid in wet, muddy or exposed nests are customarily clothed in a layer of spheres (Board et al., 1977; Board, 1980) can be taken as circumstantial evidence also in support of the hypothesis discussed by Lack. In practice, however, waterproofing is probably but one of several properties required by eggshells laid in an inimical environment; protection from microbial infection and, most especially, a defence against occlusion of the pores with mud, preening oil or nest debris warrants consideration also. Indeed Board (1982), who considered the pore system in the wider context, proposed that the pores per se ought to be considered as but one component of a diffusion pathway, the two

shell membranes and modifications of the shell surface being others, and that the last mentioned be considered as an adaptation whereby an adequate flux of respiratory gases is ensured throughout incubation even though the nest has the potential to cause progressive soiling of the shell surface.

A study of the fine structure of the naturally incubated eggshells of Helmeted Guinea Fowl (Numidia meleagris) showed that egg turning wore away spheres from the outer surface of the cuticle but that the counter-sunk nature of the outer orifice of the pore canal ensured that the majority contained a loose fitting plug of cuticle throughout incubation (Board & Perrott, 1982). As these plugs were stained, it was concluded that, through acting as filters, they protected the pore canals from contamination with nest debris and micro-organisms. The present study provided additional evidence of this role of spheres on eggshells. Thus those (Fig. 2) in the cover of incubated Grebe eggshells were smeared with amorphous material which was presumed to be of nest origin. In practice, the very limited accumulation of such material probably caused a negligible diminution of the void space in the cover. As the Grebes' nest - commonly a floating platform of vegetation having a muddy surface - leads to extensive soiling of the eggshell, as was noted in this study as well as by Tyler (1969) and Cramp & Simmons (1977) also, the question arises: what mechanism(s) ensure that the void space is not so reduced in volume that diffusion of respiratory gases is diminished? The thin, fissured layer of amorphous material on the outer surface of Grebe eggshells and bird behaviour may provide the answer. The observations that the shell becomes polished with incubation suggests that the eggs are freed of most of the contaminating material when the incubating parents turn the eggs and that only small amounts are pushed through the fissures thereby contaminating the underlying spheres. Thus it is concluded that the cover on Grebe eggshells is an adaptation which, through interaction with the brooding parent, ensures that the diffusion pathways (Board, 1982) are not occluded by nest debris.

Tullett et al. (1976) appear to be the only workers who have considered factors that may be involved with a change in the type of minerals in avian eggshells. They postulated that the incorporation of phosphorus - as phosphate - in the calcium carbonate lattice resulted in vaterite rather than calcite becoming the stable form in the cover on the calcitic shell of the eggs of the gannet, Sula bassana. They speculated that the mineral stores available to a laying bird may influence the type of calcium salt deposited in an eggshell. Thus with the gannet, a requirement for a mechanically robust skeleton to withstand the impact of a bird diving from upwards of 50 feet into the sea in pursuit of fish may limit the scope for medulary bone storage of calcium carbonate thereby increasing the importance of skeletal bone as a store and the amount of phosphate in the blood at the time of shell formation. These concepts need amendment in the light of recent investigations.

Mongin and Sauveur (1979) noted a "sharp" increase in the concentration of plasma inorganic phosphorus at the beginning but a marked diminution during the final stages of shell formation. These trends are evident also in the phosphorus content of the urine (Prashad & Edwards, 1973) but not in the eggshell. Thus with the latter, Itoh & Hatano (1964) found a phosphorus to calcium ratio of about 0.2% in the inner but one of more than 1% in the outer part of the shell. In other words, little phosphorus was deposited in the shell when the blood and urine contained the highest concentration of this element and vice versa. In an attempt to account for the very early hyperphosphataemia in laying hens, Mongin & Sauveur (1979) surmised that the calcium of bone was mobilised through the release of parathyroid hormone and that intestinal absorbtion of phosphorus increased also. A hydroxylated derivative, 1 α 25-dihydroxy vitamin D-3 ($1\alpha25\text{-}(OH)_2\text{-}D\text{-}3$), of cholecalciferol also enhances intestinal calcium transport (Holick et al., 1971; Lawson et al., 1971) and bone mobilization (Tanaka & DeLuca, 1971; Raisz et al., 1972) and may thus influence the phosphorus content of the blood. It is noteworthy also that the level of $1\alpha25\text{-}(OH)_2\text{-}D\text{-}3$, for which there is a specific binding protein in the

shell gland (Coty, 1980), in the plasma exhibits a circadian rhythm (Abe et al., 1979) such that the highest concentration of this hormone occurs at or just before the peak concentration in plasma inorganic phosphorus. Thus the available evidence suggests that hormonal control rather than the relative abundance of stores of calcium carbonate and calcium phosphate or the life style of birds determines the availability of minerals for shell formation.

The peak in the phosphorus concentration occurred in the outer part of all the eggshells included in this study (Fig. 1). It occurred in the calcitic shell of the eggs of domestic hens, in the vaterite cover on gannet eggshells and in the amorphous calcium phosphate cover on those of the Flamingo, Mallee fowl and Grebe. This evidence suggests that factors other than phosphate poisoning of calcite formation are involved in the deposition of vaterite or amorphous calcium phosphate. Indeed in their review of biomineralization, Krampitz & Witt (1978) emphasized that an interaction of an organic phase with inorganic substances was a feature common to bone and shell formation. In view of the peak in sulphur content in the cover of Grebe, Mallee fowl, Gannet and Flamingo eggshells, it is tempting to speculate that a sulphur-containing organic matrix determines the crystal forms occurring in the cover. Indeed such material may well account for the occurrence of the nut-like spheres in the outer, transition part of the calcitic shell of the Grebe eggs.

Board & Scott (1980) proposed that the term shell accessory materials ought to be used in preference to cuticle or cover to describe the outer structure of eggshells until such times as more was known about shell formation in toto. In view of the similarity of the profiles for the distribution of phosphorus (Fig. 1) across the radial plain of all the eggshells included in this study, it would seem reasonable to assume that the amorphous calcium phosphate layer on Grebe eggshells was formed in the shell gland. It is inferred also that the amorphous layer of material on the outer surface of the

Grebe eggshells is analogous to the cuticle which is laid down post-mineralization of the eggshells of domestic hens. Thus it is proposed that the term cover be retained for the stratum of vaterite (e.g. Gannet eggshells) or amorphous calcium phosphate-rich (e.g. Grebe eggshell) material which occurs on the outer surface of the calcitic shell and which is presumably formed in the shell gland per se. The term cuticle would be applied to any organic material occurring as a discrete layer on the outer surface of the cover, viz. the fissured amorphous layer on the outside of the Grebe eggshell (Plate I (c) (d)).

We wish to thank the Science and Engineering Research Council for the provision of the electron optics equipment; the Rev. B. Chapman for X-ray diffraction analysis and Professor K. Simkiss and Dr. R.S. Theobald for infra-red analysis.

REFERENCES

Abe, E., Tanabe, R., Suda, T. & Yoshiki, S. (1979). Circadian rhythm of 1 α, 25-dihydroxyvitamin D_3 production in egg-laying hens. Biochem. Biophys, Res. Comm. 88: 560-507.

Board, R.G. (1981a). The microstructure of avian eggshells, adaptive significance and practical implications in aviculture. Wildl...

Board, R.G. (1981b). A non-destructive method for studying the outer surface of avian eggshells. Br. Poult. Sci. 22: 407-408

48: 303-313.

Board, R.G. (1982). Properties of avian egg shells and their adaptive value. Biol. Rev. 57: 1-28.

Board, R.G. & Halls, N.A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. Br. Poult. Sci. 14: 69-97.

Board, R.G. & Perrott, H.R. (1979a). Vaterite, a constituent of the eggshells of the non-parasitic cuckoos, Guira guira and Crotophagi ani. Calc. Tiss. Int. 29: 63-69

Board, R.G. & Perrott, H.R. (1979b). The plugged pores of the tinamou (Tinamidae) and Jacana (Jacanidae) eggshells. Ibis, 121: 469-474.

Board, R.G. & Perrott, H.R. (1982). The fine structure of the outer surface of the incubated eggshells of the Helmeted guinea fowl (Numidia meleagris). J. Zool., Lond. 196: 445-451.

Board, R.G. & Scott, V.D. (1980). Porosity of avian eggshells. Amer. Zool. 20: 339-349.

Board, R.G., Perrott, H.R., Love, G. & Seymour, R.S. (1982). A novel pore system in the eggshells of the Mallee fowl, Leipoa ocellata. J. exp. Zool. 220: 131-136.

Board, R.G., Tullett, S.G. & Perrott, H.R. (1977). An arbitrary classification of the pore system in avian eggshells. J. Zool., Lond. 182: 251-265.

Coty, W.A. (1980). A specific, high affinity binding protein for 1 α, 25-dihydroxy vitamin D in the chick oviduct shell gland. Biochem. Biophys. Res. Comm. 90: 285-292.

Cramp, S. & Simmons, K.E.L. (1977). Editors of Handbook of the birds of Europe, the Middle East and North Africa. Vol. 1. Ostrich to Ducks. Oxford: Oxford University Press.

Holick, M.F., Schnoes, H.K., DeLuca, H.F., Suda, T. & Cousins, R.J. (1971). Isolation and identification of 1, 25 dihydroxycholecalciferol; a metabolite of Vitamin D active in intestine. Biochemistry 10: 2799-2804.

Itoh, H. & Hatano, T. (1964). Variation of magnesium and phosphorus deposition rates during eggshell formation. Poult. Sci. 43: 77-80.

Krampitz, G & Witt, W. (1978). Biochemical aspects of biomineralization. Topics in Current Chemistry. Berlin: Springer: Verlag.

Lack, D. (1968). Ecological adaptations for breeding in birds. London: Chapman & Hall.

Lawson, D.E.H., Fraser, D.R., Kodick, E., Morris, H.R. & Williams, D.H. (1971). Identification of 1, 25-dihydroxycholecalciferol: a new kidney hormone controlling calcium metabolism. Nature, Lond. 230: 228-230

Mongin, P. & Sauveur, B. (1979). Plasma inorganic phosphorus concentration during eggshell formation. Br. Poult. Sci. 20: 401-412.

Nathusius, Von W. (1882, 1887) see Tyler, C. (1964). Wilhem von Nathusius, 1821-1899, on Avian Eggshells. A translated and edited version of his work. The University: Reading.

Pooley, A.S. (1979). Ultrastructural relationships of mineral and organic matter in avian eggshells. In Scanning Electron Microscopy (Ed. Om Johari & R.P. Becker) SEM Inc. AMF O'Hare 9160666 USA.

Prashad, D.W. & Edwards, N.A. (1973). Renal excretion of calcium, phosphate and magnesium in the laying fowl. 4th European Poultry Conference, London 57-62.

Raisz, L.G., Trummel, C.L., Holick, M.F. & DeLuca, H.F. (1972) 1,25-Dyhydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. Science, N.Y. 175: 768-769.

Schmidt, W.J. (1963). Uber die Farbander in des Kalkschale der Eier von Tetrapteryx, Larus und Gavia. Sber, Ges, naturf. Freunde Bere 3 (NF): 129-143.

SCHONWETTER, M (1962). Handbuch der Oologie. Ed. W. Meise. Berlin: Akademic Verlag.

Tanaka, Y. & DeLuca, H.F. (1971. Bone mineral mobilization activity of 1, 25-dihydroxycholecalciferol. Arch. Biochem. Biophys. 146: 574-578.

Tullett, S.G., Board, R.G., Love, G., Perrott, H.R. & Scott, V.D. (1976). Vaterite deposition during eggshell formation in the cormorant, gannet and shag and in the 'shell-less' eggs of domestic fowl. Acta Zool. (Stock). 57: 79-87.

Tyler, C. (1969). A study of the eggshells of Gaviiformes, Procellariiformes, Podicipitiformes & Pelecaniformes. J. Zool., Lond. 158: 395-412.

Wedral, E.M., Vadehra, D.V. & Baker, R.C. (1974). Chemical composition of the cuticle and the inner and outer shell membranes from egg of Gallus gallus. Comp. Biochem. Biophys. 47B: 631-640.

Legend to Plates

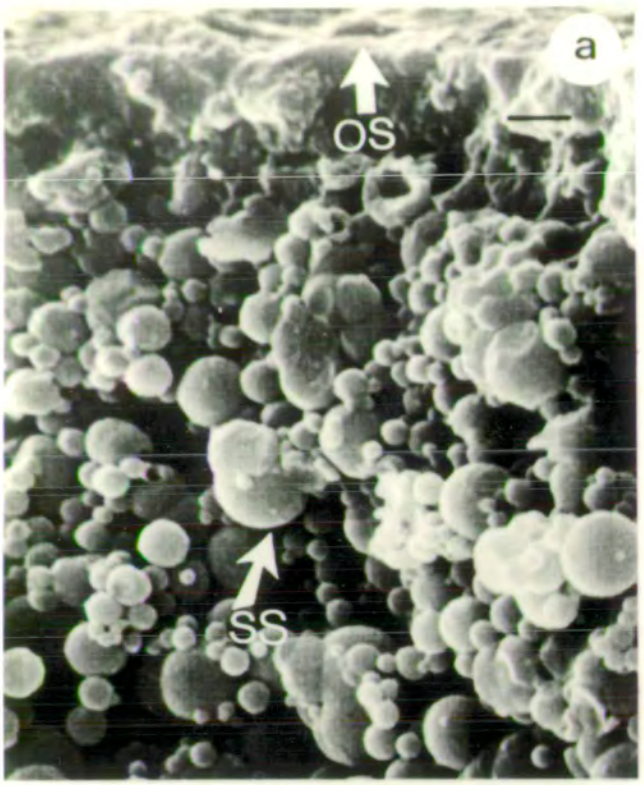
PLATE I. (a) A stylized drawing of a radial section of the eggshell of the Great Crested Grebe, Podiceps cristatus; (b) Electron micrograph of a radial section of Great Crested Grebe eggshell; bar marker, 100μm; (c) Outer surface of unincubated egg; bar marker, 100μm and (d) outer surface of incubated eggshell; bar marker, 100μm. BC, basal caps; CO, cone; C, crack; CL, cone layer; F, fissure; P, plaques; PC, pore canal; SM, shell membranes; SS, surface stratum, and TL, transition layer.

PLATE II Electron micrographs of radial sections of the eggs shells of Great Crested Grebe, Podiceps cristatus. (a) Surface stratum of untreated eggshell; bar marker, 1μm; (b) surface stratum of eggshell which had been boiled in 5% (w/v) sodium hydroxide for 5 minutes; bar marker, 10μm; (c) spheres in the surface stratum of unincubated egg; bar marker, 1μm, and (d) spheres in surface stratum of incubated egg; bar marker, 1μm. FF, flat face of sphere; FS, fused spheres in outer stratum of spheres (SS); OS, outer surface; P, particulate matter; PC, pore canal; PP, pore plug.

PLATE III Electron micrographs of the transition layer [see Plate I(a)] of the shell of the Great Crested Grebe, Podiceps cristatus. (a) and (b) radial sections showing entrapped spheres (ES) in a crystalline layer (CL) overlaid with the stratum of spheres (SS); bar markers 10 and 1μm respectively; (c) Details of the composite spheres showing covers (C) central aggregate of spherules (AS) and finger-like projections (P), bar marker 1μm; (d) Details of a fractured (F) central aggregate of spherules; bar marker, 1μm.

PLATE IV Electron micrographs of the eggshells of the Great Crested Grebe (Podiceps cristatus). (a) Plastic embedded (P) cover showing the honey-combed structure remaining after treatment with carbonated water (bar marker, 10μm); E, is the highly corroded transition layer (see PLATE 1a) and A a crack between the transition layer and the main part of the shell. (b) Inner surface of eggshell, after boiling in NaOH, showing tips (T) of cones and inner orifice (P) of pore canal; bar marker, 100μm. (c) Radial section of shell showing cone layer (CL), inner pore orifice (PO) and shell (SM) membranes; bar marker, 100μm. (d) Details of cone layer, as seen in radial section of shell freed of shell membranes by bliling NaOH (bar marker = 10μm), T, tip, N, neck and B, base of cone.

PLATE V Electron-micrographs of inner surface of the incubated eggs of Great Crested Grebe (Podiceps cristatus). (a) Inner surface of shell showing the fibres (F) of the shell membranes and the ruptured tips (RT) of the cones (bar marker = 20μm). (b) The ruptured tip of the cone attached to the fibrous (F) shell membrane showing the membrane (M) bounded by central vacuole (CV) surrounded in turn by satellite vesicles (SV) and crystals with a radial symmetry (RS) and corroded (CE) periphery; bar marker, 10μm. (c) The fibrous shell membrane (F) bearing many ruptured tips (RT) of the cones; bar marker, 50μm. (d) Radial fracture of cone showing large vesicle (V), crystals with radial (RS) and oblique (O) symmetry; bar marker, 10μm.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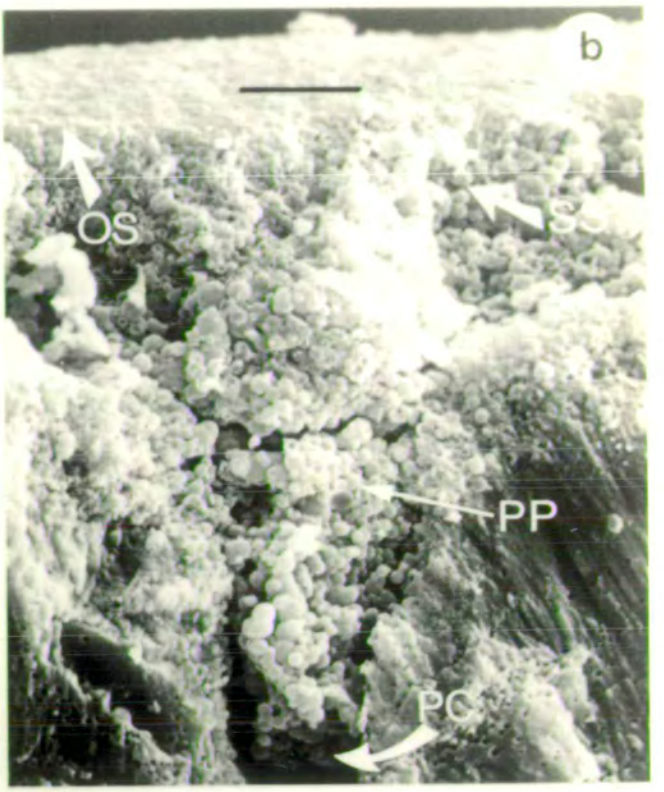
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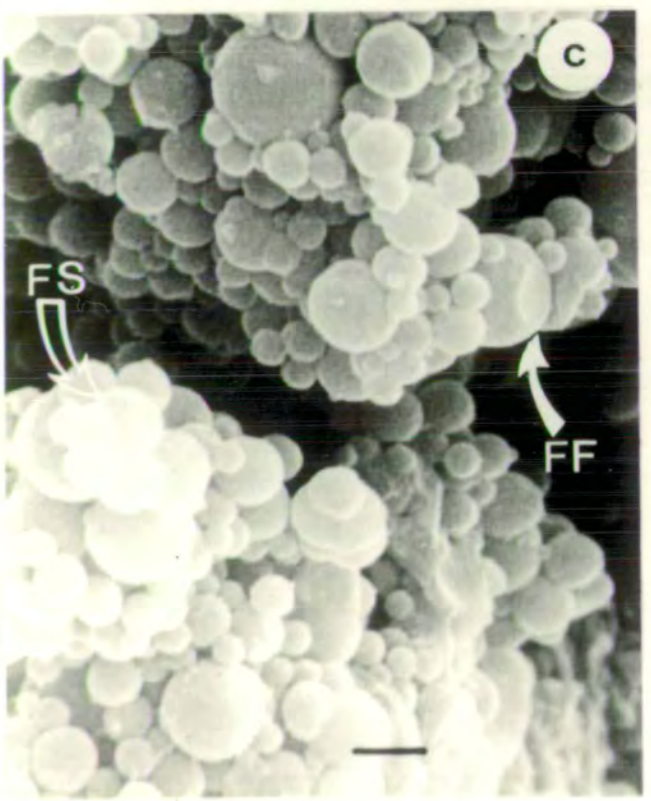
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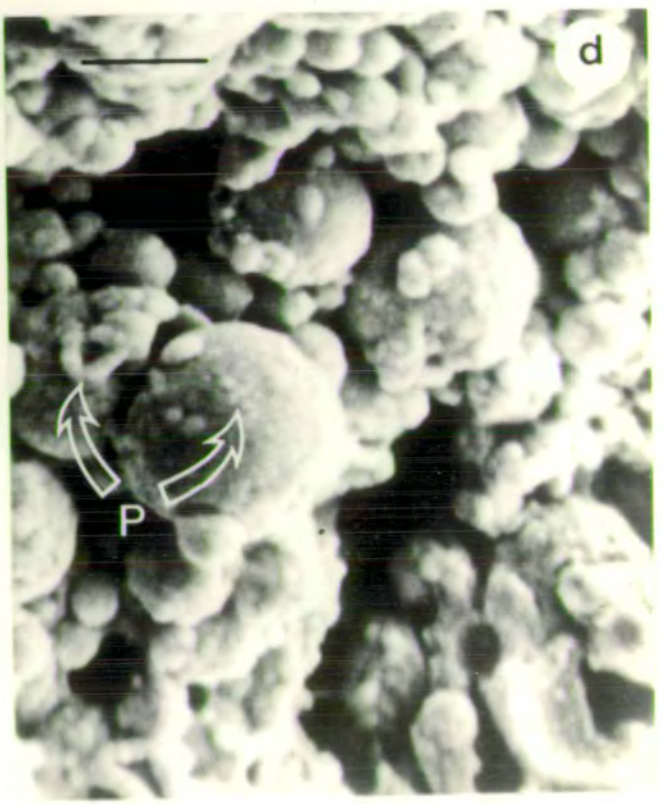
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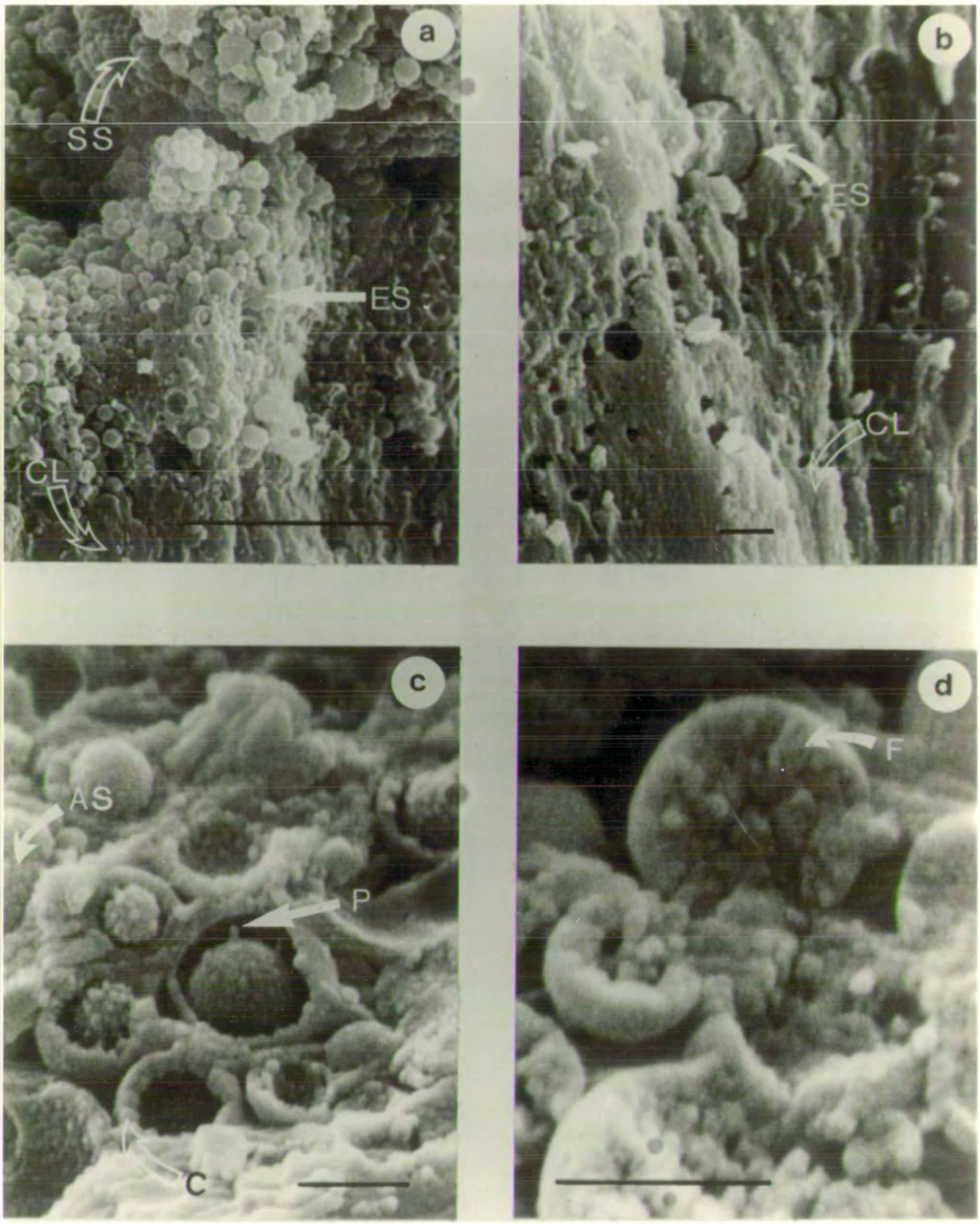
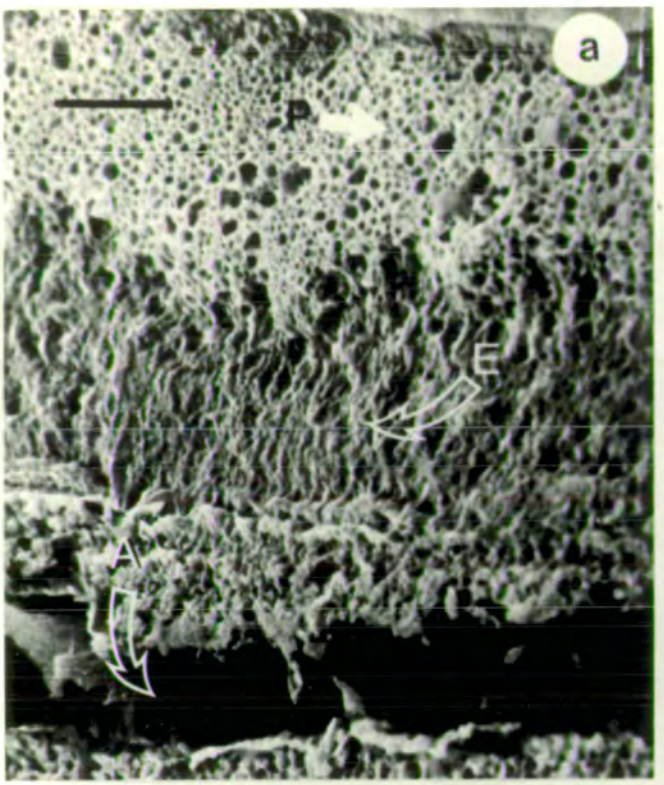
Plate III

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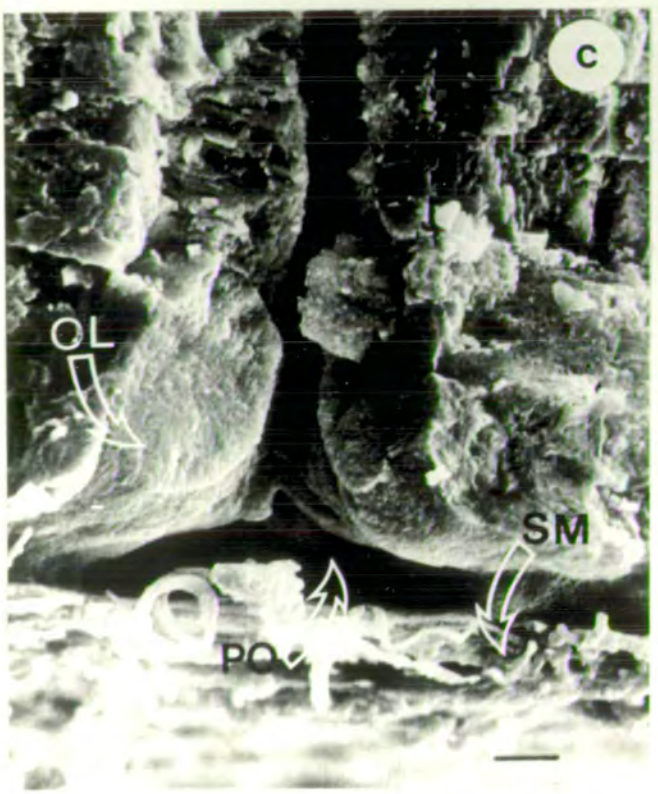
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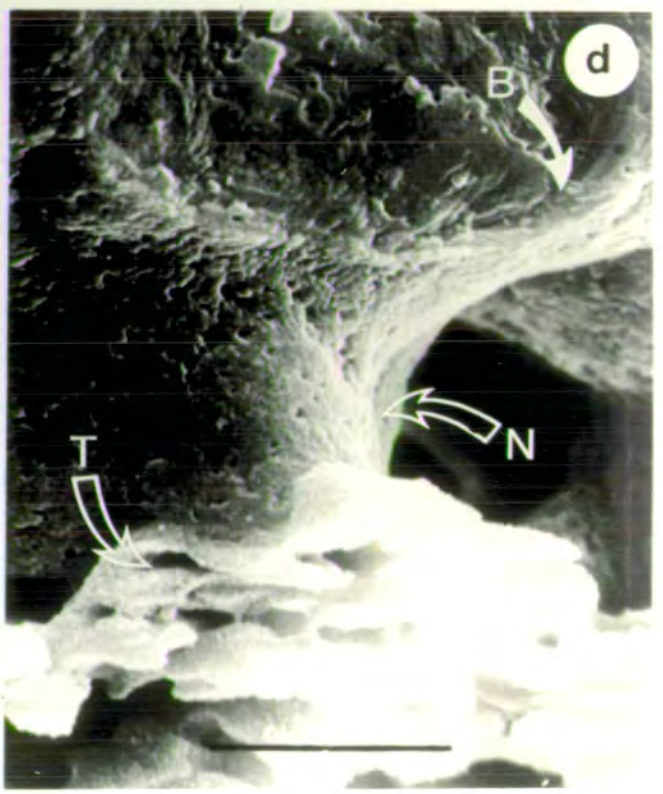
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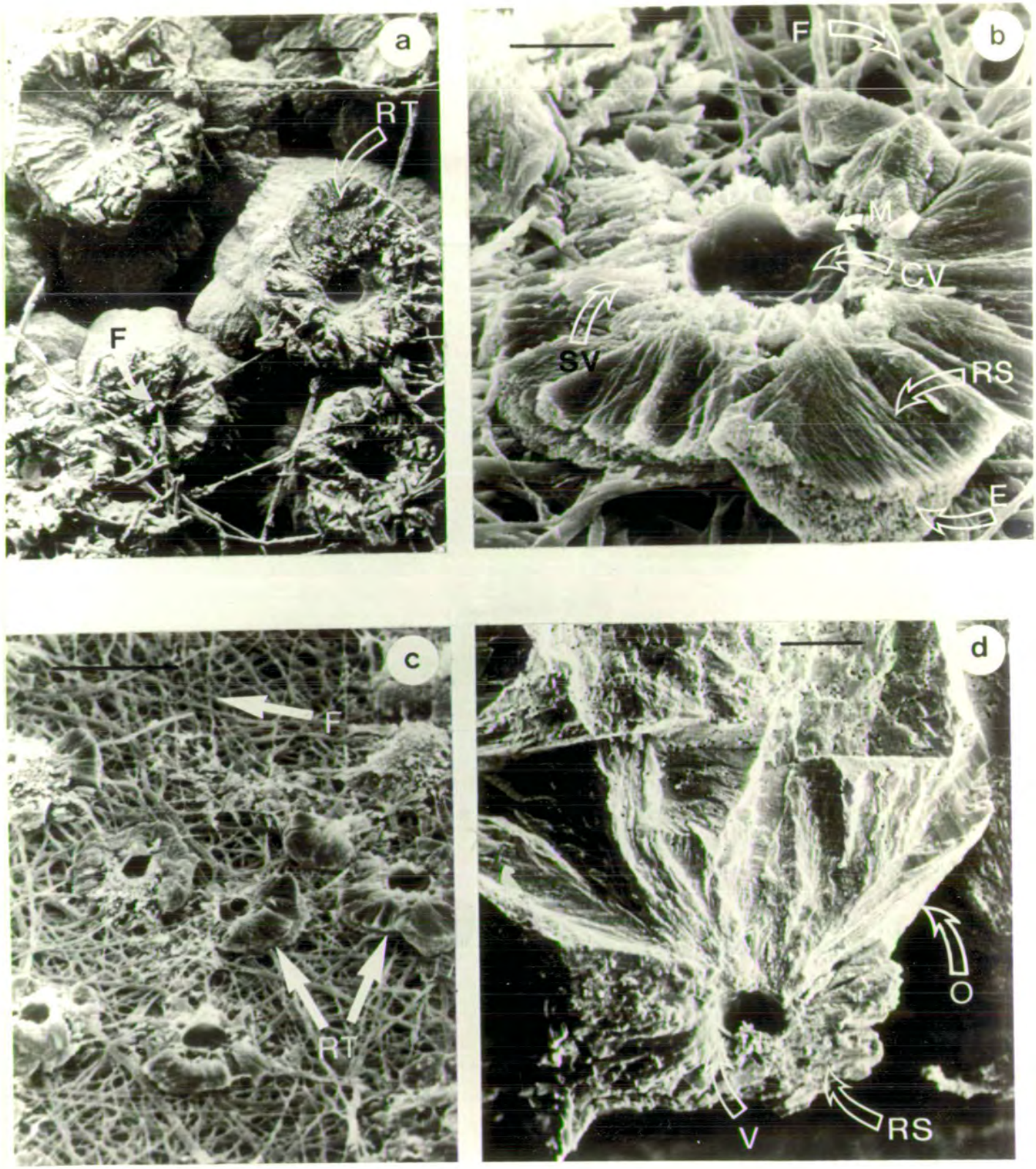
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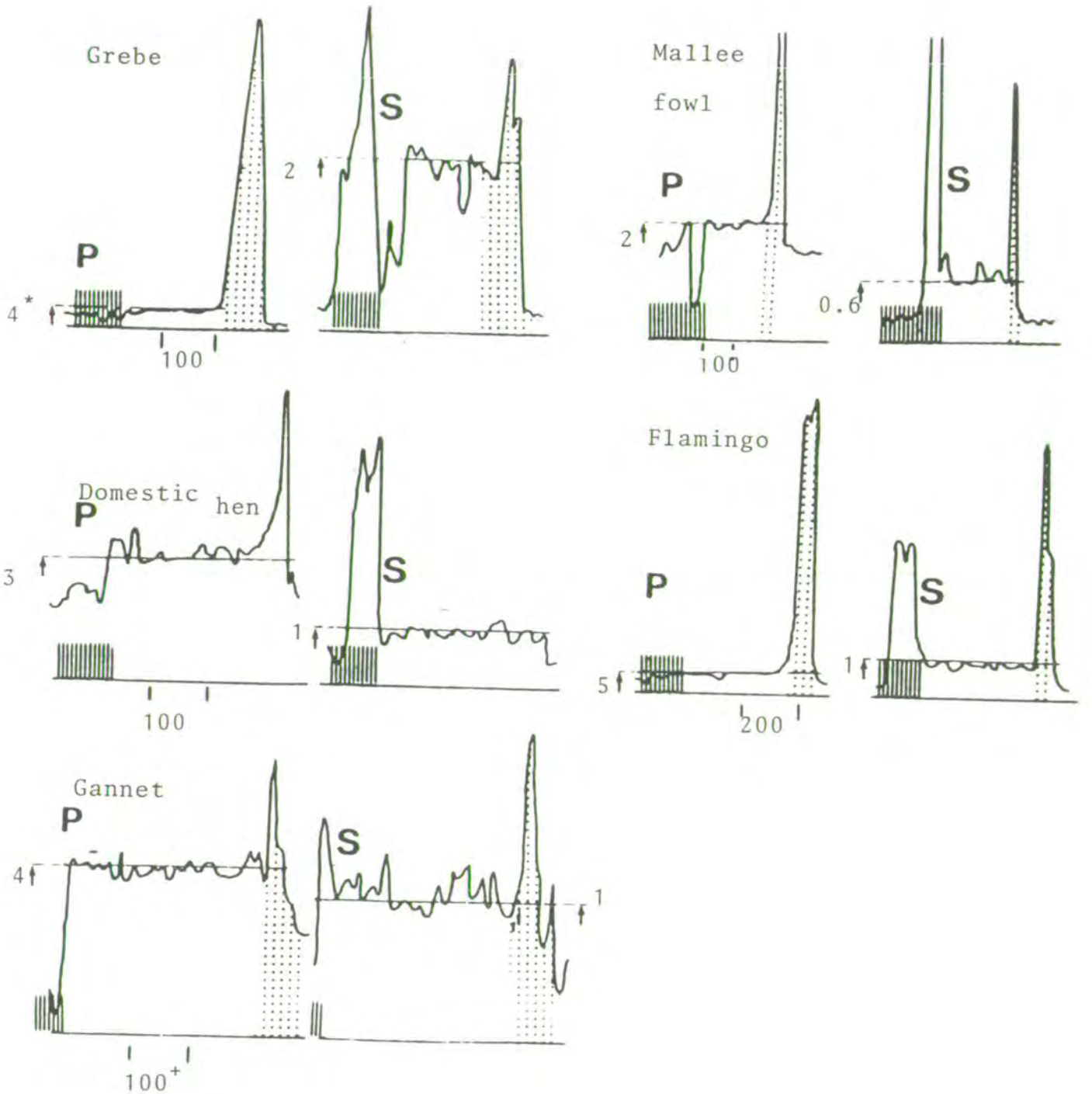
Fig.1. The polished radial face of gold-sputtered eggshells in plastic was analysed (15kV 0.2 μA) in a JEOL JCXA-733 Electron Probe Analyser the spectrometer of which was calibrated for sulphur and phosphorus. The movement of the specimen was computer controlled such that a stepped-scan was done, 10μm intervals between 10 sec samplings, P = phosphorus, S = sulphur; solid parallel lines show the position of shell membranes; dotted lines the cover.

* The concentration (%) of element is expressed as a percentage of the reading obtained with the pure sample used to calibrate the instrument.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Domestic hen
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Flamingo
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1
200
Gannet
P
S
4
1
100+

RAPID COMMUNICATION

A Novel Pore System in the Eggshells of the Mallee Fowl, *Leipoa ocellata*

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ABSTRACT The outer orifice of the funnel-shaped pores in the eggshell of the mallee fowl, *Leipoa ocellata*, contain irregularly shaped pieces of crystalline material. The whole of the outer surface of the shell is covered with spheres containing amorphous calcium phosphate but little organic material. Thus they would not be expected to support microbial growth and they would not be corroded by organic acids produced by microorganisms in the incubation mounds.

A detailed study of the relationships of oxygen consumption, water loss during incubation, the eggshell's water vapor conductance, porosity, egg mass, and incubation period led Rahn and Ar ('80) to conclude that 1) "all these functions are proportional to the product of egg mass and the rate of development where the latter is defined as the inverse of time," and 2) "these interrelationships account at the end of incubation for similar O_2 and CO_2 tensions in the air space of eggs, utilization of calories (0.5 kcal$\cdot g^{-1}$) and water loss (0.15 g$\cdot g^{-1}$)." It is implicit in these two statements that certain eggshells are adapted to unusual incubation conditions, e.g., high humidity of the nest atmosphere. Thus Birchard and Kilgore ('80) contend that such an adaptation is a feature of the eggshells of the tunnel-nesting bank swallow (*Riparia riparia*), a species whose shell has a greater conductance than those of a related species, the open nesting barn swallow (*Hirunda rustica*). A humid atmosphere is a feature also of the incubation mounds of megapodes (Seymour and Ackerman, '80). Some of these birds use heat produced by aerobic microorganisms that break down organic material in the mounds. High humidity in the mound limits its water loss from the eggs, and the fixed air space, common to other birds, is not formed (Seymour and Rahn, '78; Vleck et al., '80). The eggshells have water vapor conductances considerably greater than predicted mainly as a consequence of their relative thinness. This is no doubt an adaptation so that the embryo can exchange respiratory gases with an atmosphere enriched with CO_2, but depleted in respect of O_2 (Seymour and Ackerman, '80).

Because of the high humidity and microbial activity of the mounds, it may be presumed that megapode eggshells have become adapted to avoid colonization by microorganisms. The glycoprotein cuticle of the eggshell of the domestic hen is digested by bacteria and colonized by fungi when stored under humid conditions (Board et al., '79) and, actinomycetes can grow on eggshells under museum conditions (Board and Perrott, '79). The present study of the eggs of the mallee fowl (*Leipoa ocellata*) was concerned with the outer surface of the eggshell because of its possible adaptation to counter what appears to be a potentially hostile environment of the mound.

MATERIALS AND METHODS

The radial face of pieces of fractured shells of mallee fowl eggs were sputter-coated in vacuo with gold and examined with a JEOL 35C scanning electron microscope. Other pieces of shell were embedded in plastic (Plasticraft Research Ltd., Leeds, England) and polished with 0.5 μm diamond paste on the radial faces. These were then coated with gold and examined with a JEOL JXA 50A electron probe microanalyser. The X-ray results (Fig. 3) obtained from radial scans for magnesium were recorded on a chart moving at known speed. The same instrument fitted with an EDAX energy dispersive analyser was used to carry out spot analyses of elements on uncoated eggshells. The crystallography of pieces of whole or

powdered (affixed to glass fibre with silicon grease) shell was studied, using copper Kα irradiation, with a Phillips X-ray Powder Diffractometer (PW 1050) and the Debye Scherrer method (Cullity, '56) respectively. Powdered shell in a Nujol (a mineral oil) mull (paste) was investigated with infrared radiation, using a Perkin Elmer 197 spectrophotometer for calcium phosphate.

RESULTS

The pores in the mallee fowl contained irregularly shaped pieces of crystalline material (presumed to be calcite), and the entire outer surface of the shell was covered with spheres (Fig. 1). The latter, which were not removed from the shell by boiling in 5% (w/v) aqueous sodium hydroxide, and the outer edge of the shell contained appreciable amounts of phosphorous (Fig. 2), an element that was not detected elsewhere in the shell. The spheres contained calcium phosphate (infrared analysis) in an amorphous form (no patterns given with X-ray diffraction). The eggshells of other megapodes (*Megapodius freycinet, Alectura lathami,* and an unidentified museum specimen) had unplugged, funnel-shaped pores the outer orifice of which was covered with spheres rich in calcium phosphate. All the megapode eggshells showed a common pattern of Mg^{2+} distribution across the radial plane (Fig. 3).

DISCUSSION

Mallee fowl eggshells are characterized by a pore system (capped and plugged) that is not in the classifications proposed by Board ('80) or Board et al. ('77). It was noted above that there are a priori reasons to expect adaptations of the megapode eggshell so that their surfaces would not be colonised by microorganisms. The resistance of the spheres to boiling NaOH indicates that they contain little if any organic material. Moreover, their content of amorphous calcium phosphate, a relatively insoluble salt vis-à-vis calcium carbonate in calcite form, could be expected to protect the underlying shell from corrosion due to acids arising from microbial breakdown of organic material in the mound. Progressive corrosion due to such acids is a feature of alligator (*Alligator mississipiensis*) eggs incubated in mounds of vegetation (Ferguson, '81). It has been proposed (Board '80) that spheres on the outer surface of avian eggshells protect the pores from flooding or blockage with mud, nest debris, or preening oils. A study of the incubated eggs of the helmeted guinea fowl (*Numidia meleagris*) has shown that spheres are lost from the outer surface of the cuticle, presumably as a result of egg turning, but that those in the orifice of the pore canals are protected by the ridges on the shell (Board and Perrott, '82). The spaces between the spheres at the surface of the cuticle became filled with debris, but radial fissures in the plugs in the pore orifice permitted gaseous diffusion. Although megapode eggs would not be exposed to all of the inimical features noted above, they may well have an obligate requirement for an outer stratum of spheres so that the pore canals are not occluded with debris during protracted incubation in mounds composed of sand and vegetation. As yet, however, no reasons can be advanced to account for the plugs in the pore orifice of the mallee fowl.

In the radial plane of the eggshells of ten species of the Galliformes there are two peaks in the concentration of Mg^{2+}, one at the inner (cone) layer and the other at the outer surface of the shell (Board and Love, '80). Although assigned to this order, megapodes produce shells with only one peak of Mg^{2+} (in the cone layer). This was a common feature of more than 60 species belonging to 19 orders other than Galliformes. From a regression analysis of the width of the inner band of the Mg^{2+}-rich shell against total shell thickness, Board and Love ('80) speculated that analysis of the radial distribution of this element may indicate the relative length of time that an egg spends in the distal part of the isthmus, in which shell formation is initiated (Wyburn et al., '73), and in the shell gland wherein bulk mineralization occurs. Our analysis may indicate a short residence time in the shell gland and hence account for the relative thinness and high conductance of megapode eggshells.

Fig. 1. Radial section of the eggshell of mallee fowl (*Leipoa ocellata*) showing the pore canal (PC) with a plug of crystalline material (P) in its outer orifice. The surface of the shell is covered with a layer of spheres (S). The lumen of the pore canal vents via fissures (F) in the pore plug to the void spaces in the sphere layer. Bar marker = 50 μm.

Fig. 2. Results obtained with an EDAX energy dispersive analyser from a survey of the distribution of phosphorous in the sphere layer (a), the outer edge (b), and centre (c) of the calcitic shell of the mallee fowl.

Fig. 3. The radial distribution of the mallee fowl eggshell determined with an electron probe microanalyser. The major concentration of the element occurred in the cone layer.

ACKNOWLEDGMENTS

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LITERATURE CITED

Birchard, G.F., and D.L. Kilgore (1980) Conductance of water vapor in eggs of burrowing and non-burrowing birds: Implications for embryonic gas exchange. Physiol. Zool., *53*:284–292.

Board, R.G. (1980) The avian eggshell – A resistance network. J. Appl. Bacteriol., *48*:303–313.

Board, R.G., and G. Love (1980) Magnesium distribution in avian eggshells. Comp. Biochem. Physiol., *66A*:667–672.

Board, R.G., and H.R. Perrott (1979) A note on actinomycetes on a museum specimen of eggshell. J. Appl. Bacteriol., *47*:539–541.

Board, R.G., and H.R. Perrott (1982) The fine structure of the incubated eggshells of the helmeted guinea fowl (*Numidia meleagris*). J. Zool., (Lond.) (in press).

Board, R.G., S. Losebey, and V.R. Miles (1979) A note on microbial growth on hen eggshells. Br. Poult. Sci., *20*:413–420.

Board, R.G., S.G. Tullett, and H.R. Perrott (1977) An arbitrary classification of the pore system in avian eggshells. J. Zool. (Lond.), *182*:251–265.

Cullity, B.D. (1956) Elements of X-ray Diffraction. Addison-Wesley, Reading, Massachusetts.

Ferguson, M.W.J. (1981) Increasing porosity of the incubating alligator eggshell caused by extrinsic microbial degradation. Experientia, *37*:252–254.

Rahn, H., and A. Ar (1980) Gas exchange of the avian egg: Time, structure and function. Am. Zool., *20*:477–484.

Seymour, R.S., and R.A. Ackerman (1980) Adaptations to underground nesting in birds and reptiles. Am. Zool., *20*:437–447.

Seymour, R.S., and H. Rahn (1978) Gas conductance in the eggshell of the mound-building brush turkey. In: Respiratory Function in Birds, Adult and Embryonic. J. Piiper, ed. Springer-Verlag, Berlin, Heidelberg, pp. 243–246.

Vleck, D., C.M. Vleck, and R.S. Seymour (1980) Megapode eggs: Energy and water loss during incubation. Am. Zool., *20*:906.

Wyburn, G.H., H.S. Johnston, M.H. Draper, and M.F. Davidson (1973) The ultrastructure of the shell forming region of the oviduct and the development of the shell of *Gallus domesticus*. Q. J. Exp. Physiol., *58*:143–151.


S
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PC
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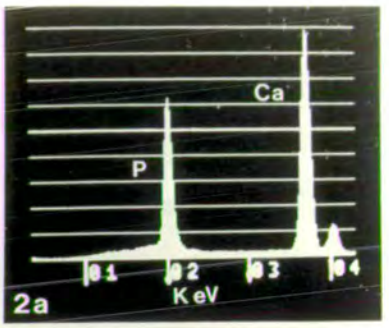
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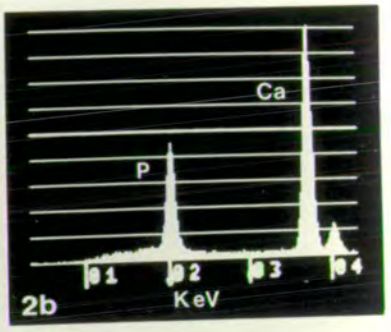
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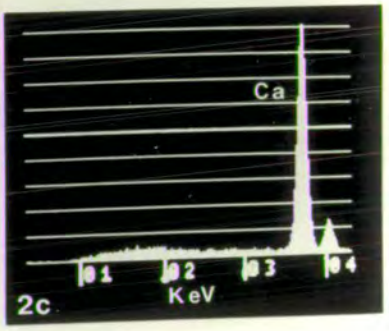
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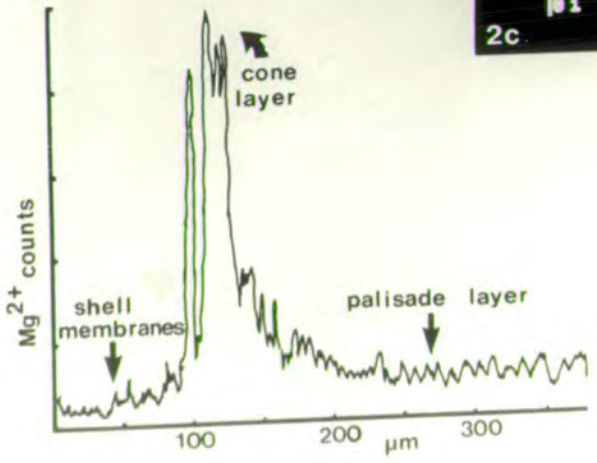
cone layer
Mg^{2+} counts
shell membranes
palisade layer
100
200
μm
300

3

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Determinants of avian eggshell porosity

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(With 5 figures in the text)

The manner in which the porosity of avian eggshells varies with egg weight is discussed in relation to the number of pores, their cross-sectional area and length. Shell porosity and ways in which this can change to satisfy different environmental demands are discussed on the basis of a simple model which envisages the shell as being composed of columns of calcite.

Contents

Introduction

Gaseous exchange across the avian eggshell is brought about by diffusion (Wangensteen, Wilson & Rahn, 1970/71). Oxygen diffusing in or carbon dioxide and water vapour out must cross a number of resistances arranged in series (see Tullett & Board, 1976), the most important being that imposed by the geometry of the pore canal (Ar, Paganelli, Reeves, Greene & Rahn, 1974).

The permeability of the shell to water vapour, i.e. its conductance, has been shown to be species specific and rather precisely correlated not only with the incubation period and weight of the egg (Rahn & Ar, 1974) but also with the rate of water loss during incubation (Drent, 1970; Rahn & Ar, 1974) and the metabolic rate near hatching time (Rahn, Paganelli & Ar, 1974). A relationship between the shell conductance to water vapour (G_{H_2O}, in mg/day/Torr water vapour pressure difference across the shell) and fresh egg weight (W, in g) has been derived (Ar *et al.*, 1974) from a linear regression analysis of log G on log W. It can be expressed as the power function:

$$G_{H_2O}=0{\cdot}432\times W^{0{\cdot}780} \quad (1)$$

By modifying Fick's first law of diffusion and applying suitable substitutions Ar *et al.* (1974) have shown that the conductance to water vapour is proportional to the area available for diffusion (A_p, the total functional pore area in cm^2) and inversely proportional to pore length or shell thickness (L, in cm) according to the equation:

$$G_{H_2O}=23{\cdot}42\ \frac{A_p}{L} \quad (2)$$

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Substitution of Equation (1) into Equation (2) gives an expression of A_p and L as a function of egg weight:

$$\frac{A_p}{L} = 0{\cdot}018\ W^{0{\cdot}780} \tag{3}$$

As pointed out by Ar *et al.* (1974), Equation (3) represents the geometrical factors of the shell that determine the conductance for all gases which exchange across the shell. The variation in shell thickness with egg weight has been investigated (Ar *et al.*, 1974) and can be expressed by the regression equation:

$$L = 5{\cdot}126 \times 10^{-3}\ .\ W^{0{\cdot}456} \tag{4}$$

where L=shell thickness (cm).

Substitution of Equation (4) into Equation (3) gives an expression for the total functional pore area, A_p, as a function of egg weight:

$$A_p = 9{\cdot}2 \times 10^{-5}\ .\ W^{1{\cdot}236} \tag{5}$$

This latter equation embodies two further components of shell porosity since the term A_p is the product of the total number of pores and the area of each pore. Ar *et al.* (1974) posed the question as to how the predicted increase in total functional pore area with egg weight is brought about—is it achieved by increasing the number of pores or their diameter or some combination of the two?

In the present paper an answer is given to this question and two other aspects of shell structure which have a functional relationship are examined, these being the relationships between the number of pores per unit area and egg weight and between the number of cones per unit area and shell thickness. The results obtained are discussed in terms of a simple shell model in which the basic building blocks of the shell, the columns of calcite, are the major considerations.

Materials and methods

Source of eggs

The eggs used by Tullett (1975) were re-examined together with additional material from the same sources and others listed in the acknowledgements. Some of the eggs used by Ar *et al.* (1974) and others investigated subsequently were kindly donated by Professor H. Rahn (State University of New York at Buffalo).

The methods used were essentially those of Tullett (1975) with the following additions.

Assessment of the number of pores per egg

Determinations of pores/cm^2 were transposed into pores per egg through multiplication by the surface area of the egg. The surface area (cm^2) was calculated from egg weights (given either by Lack (1968), Ar *et al.* (1974), Rahn (pers. comm.), or independently determined) according to the equation:

$$\text{Surface area} = 4{\cdot}917\ .\ W^{0{\cdot}661} \tag{6}$$

where W is the egg weight (g) (Rahn, pers. comm.).

Assessment of the area of individual pores

The majority of pores are funnel-shaped, the wider orifice being outermost. It was surmized therefore that a good assessment may be made of pore area by determining the cross-sectional area at its smallest part, i.e. in the inner layer of the palisade region.

Fragments of eggshell were taken from the shoulder of the eggs and their membranes removed by boiling in 5% (w/v) NaOH. The inner surface was examined by scanning electron microscopy and tracings of the pore outlines made directly from the viewing screen. These were later cut out and their area measured with an automatic area meter (Hayashi Denko Co. Ltd., Tokyo). At least 10 pores per shell were examined in this way and the individual pore area calculated to the nearest μm^2.

Shell thickness

Shell thickness of fragments taken from the shoulder of eggs was measured using a screw micrometer having hemispherical anvils. The measurements were made after the membranes/cuticle were removed by boiling in NaOH or, in the case of eggs with covers (see Tullett, Board, Love, Perrott & Scott, 1976), after the membranes had been removed in the above manner and the cover scraped off.

Results

A least square function for log pores per egg on log egg weight yielded an egg weight power function that was essentially similar for data derived either from the shoulder or blunt pole of the egg. The composite shown in Fig. 1 includes data from 59 species and is expressed by the equation:

$$\text{Pores per egg} = 1449 \,.\, W^{0.420} \tag{7}$$

where W is the egg weight in g. The correlation coefficient is high ($r = 0.853$) and significant ($P \ll 0.001$).

Similarly, individual pore area (μm) has been plotted against egg weight on a double logarithmic grid (Fig. 2). The regression line is represented by the equation:

$$\text{Individual pore area} = 5.425 \,.\, W^{0.804} \tag{8}$$

The correlation coefficient is high ($r = 0.950$) and significant ($P \ll 0.001$).

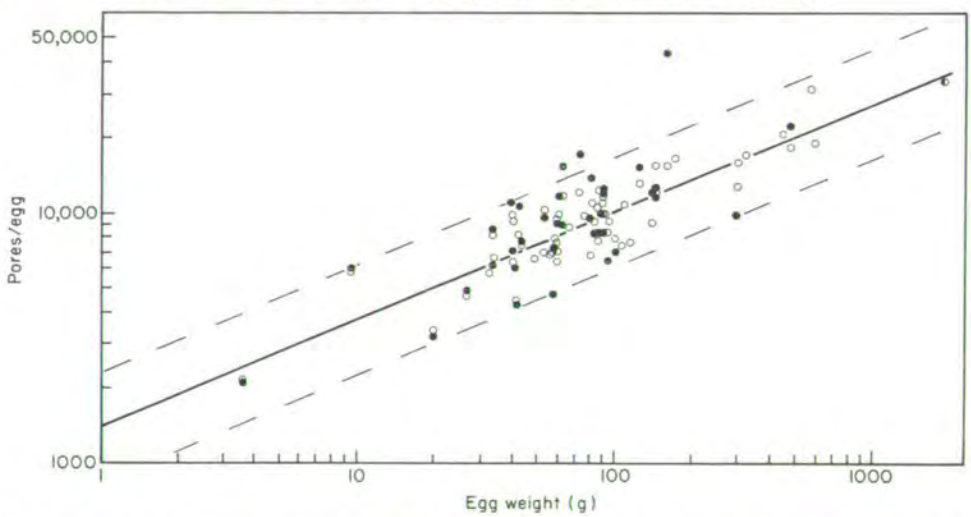

FIG. 1. Pores per egg plotted against egg weight (g), $n = 94$. No. of spp. = 59. Solid line is the regression curve, the pecked lines enclose ±2 S.E. of the estimate and represent the 95% confidence limits for the log of pores per egg. The regression lines for data derived from the shoulder (○) and blunt pole (●) were similar; the composite for all the data is shown. Star plot is for Common loon. Scale, log–log.

Eggshells of the Shag (*Phalacrocorax aristotelis*) posed a problem in estimating pore area. Boiling in NaOH opened up the pores to a great extent. It is not known whether this was due to removal of calcite or perhaps protein from between the cones/columns. Thus, in a "minimum-boiled" shell, some pores were measured which were only slightly larger (150–200 μm²) than normal for eggs of this weight while others were etched to such an extent that their area was 350–550 μm². It was for this reason that the Shag was not included in the analysis for the regression line. Eggshells of the Cormorant (*Phalacrocorax*

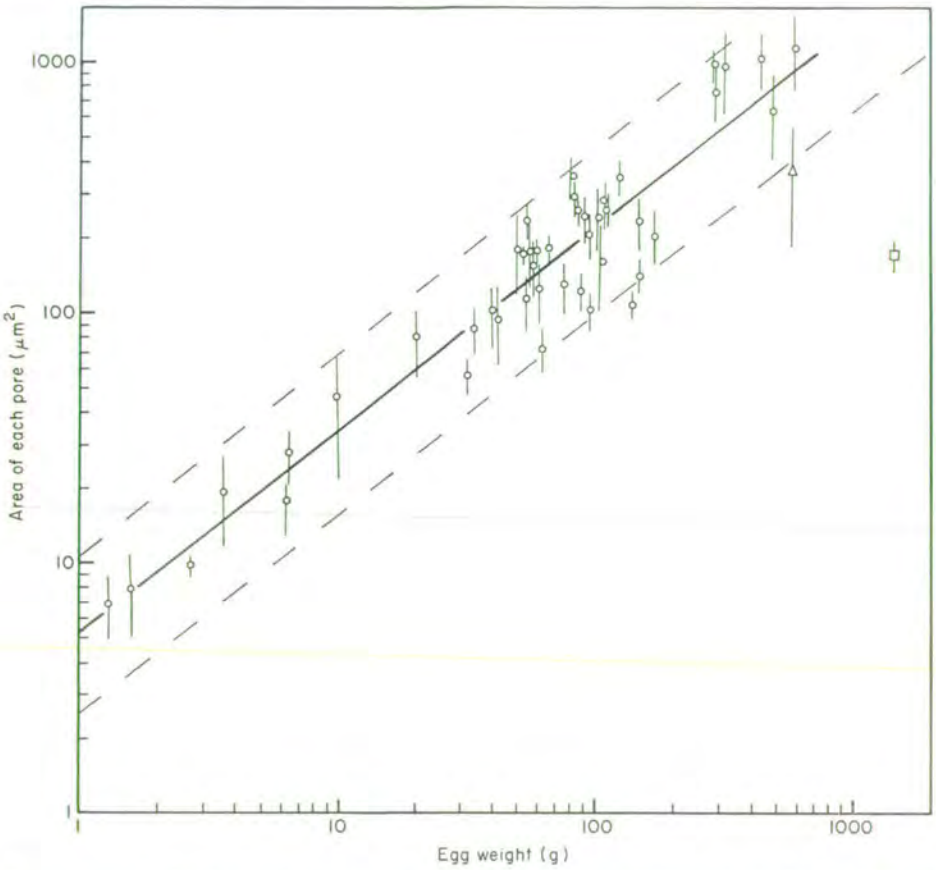

FIG. 2. Regression of individual pore area (μm²) on egg weight (g). Regression curve and pecked lines as in Fig. 1. The points represent the mean of at least 10 independent estimates of pore area on shell fragments from the shoulder of the eggs and the vertical bars indicate ± one standard deviation. (△) is Emu plot, (□) is Ostrich. No. of spp. = 45. Scale, log–log.

carbo) and Gannet (*Sula bassana*), which may be expected to have a similar composition (Tullett, Board, Love *et al.*, 1976), did not exhibit this range and were included.

Data for the Emu (*Dromaius novaehollandiae*) and Ostrich (*Struthio camelus*) are also shown in Fig. 2 but they were not included in the regression analysis as the eggs of both have an unusual pore arrangement. In the case of the Ostrich, pores originating in cone layer branch repeatedly, some linking up again, as they traverse the shell to the outside.

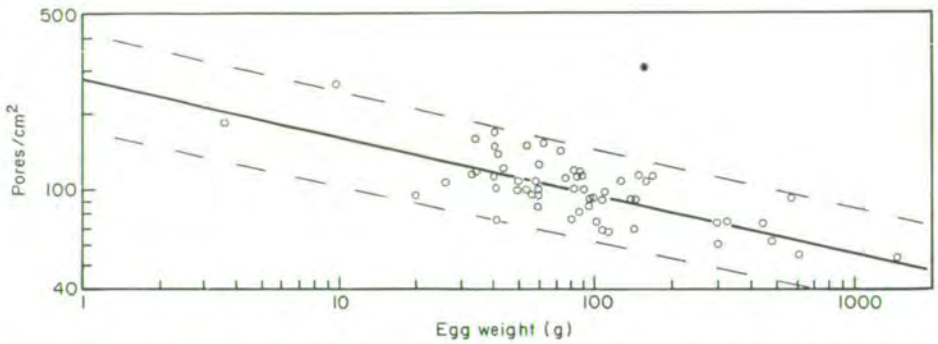

FIG. 3. Pores/cm² at the shoulder of the egg against egg weight (g). Lines as in previous figures. Star plot is for Common loon. No. of spp.=58. Scale, log–log.

In terms of the diffusion pathway the effect of this branching is to increase progressively the area available for diffusion as the pore system passes from the cone layer to the outer surface of the eggshell. The manner of the branching must therefore increase the pore area measured at the cone layer/palisade layer interface up to the empirically determined functional pore area. The Emu pore arrangement is more complex (Board & Tullett, 1975), pores traversing the shell often branch (which will increase the area available for diffusion) before entering a labyrinth of tubules running just under the outer surface of the shell. The effect of this labyrinth on gaseous exchange and water loss is not known.

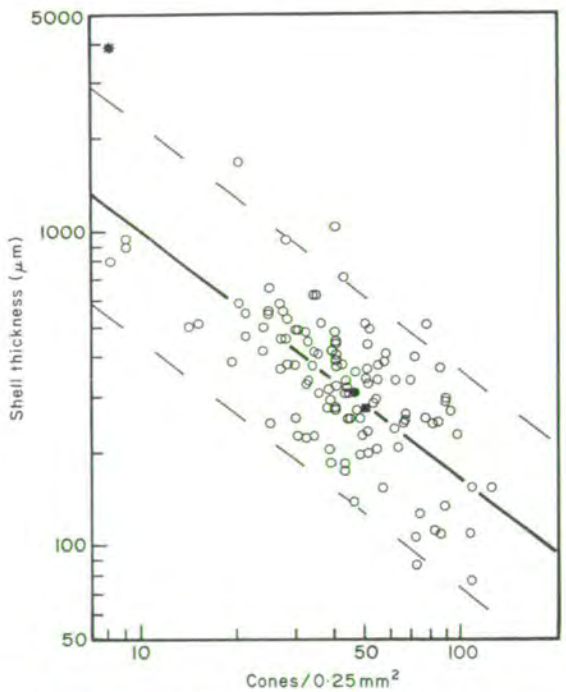

FIG. 4. Shell thickness (μm) against cones/0·25 mm². Lines as in previous figures. Star plot is for Aepyornis. (●) Two plots coincide. (■) Three plots coincide. No. of spp.=123. Scale, log–log.

The number of pores/cm^2 were found to be related to egg weight (Fig. 3) according to the equation:

$$\text{Pores/cm}^2 = 279{\cdot}65 \, . \, W^{-0{\cdot}231} \tag{9}$$

The regression coefficient is high ($r = -0{\cdot}736$) and significant ($P \ll 0{\cdot}001$).

Since the eggshell can be considered as columns of calcite (see Tullett, 1975) it may be expected that ones of greater diameter grow to a greater length. Cones/0·25 mm^2 was used as an index of column diameter and was found to be related to shell thickness (Fig. 4) according to the equation:

$$\text{Shell thickness } (\mu\text{m}) = 5948 \text{ cones}/0{\cdot}25 \text{ mm}^{2\,-0{\cdot}781} \tag{10}$$

The correlation coefficient is high ($r = -0{\cdot}704$) and significant ($P \ll 0{\cdot}001$).

Data for the Common loon (*Gavia immer*) have been included in Figs 1, 3 (star plots) and 4. It was not included in the regression analyses for Figs 1 and 3 but is shown because it has been found that in relation to the egg weight (*c.* 154 g) the water conductance of the

FIG. 5. Schematic representation of a transverse section through a "larger than normal" pore typical of the Common loon.

shell is very high (*c.* 98 mg/day/Torr; (Rahn, pers. comm.)). The number of cones/0·25 mm^2 for this eggshell was found to be about 27 which according to Tullett (1975) should give about 76 pores/cm^2. Actual pore counts gave a figure of 307. Scanning electron microscopy revealed that although the "pores" were situated between the cones not all conformed to the usual pore orifices described by Tullett (1975). For example, although pores of the type described by Tullett (1975) were found (area about 600 μm^2) others extended as small cracks between many cones/columns (Fig. 5) and had cross-sectional areas ranging from about 1000 to 6000 μm^2 at the cone layer/palisade layer interface.

Discussion

The geometry of the pore canals determines the conductance for gases which exchange across the eggshell. Shell conductance has two components, pore length and total pore area, both of which are related to egg weight (Ar *et al.*, 1974). In the present communication the two components of the total functional pore area (A_p) i.e., the total number of pores per egg and the area of each pore, have been shown to be independently related to egg weight (Equations 7 and 8). The product of these two equations is:

$$A_p = 7{\cdot}86 \times 10^{-5} \, . \, W^{1{\cdot}224} \tag{11}$$

Although there is good agreement between this equation and Equation (5) the former has a slightly lower constant. This may arise because measurements of the pore area were

made in the inner layer of the palisade region at the narrowest part of the pore. As pointed out by Steen (1971), the pores widen out considerably at the outer surface of the shell thereby increasing the diffusion area between the atmosphere and the gases in the mouth of the pore itself.

Equation (8) is an expression for the rate-limiting pore area rather than the functional pore area, and for a given pore area in the inner layer of the palisade region there will be a certain variability in the rate of diffusion of gases along the pores depending on the degree of widening and/or branching on the pore canal as it crosses the shell. During incubation calcium is resorbed from the eggshell for use by the embryo but it is not known whether this affects the cross-sectional of the pores as measured at the cone layer/palisade layer interface.

Cone density has been shown to determine pore density (Tullett, 1975). In the present communication data have been presented (Fig. 4) which suggests that it also determines shell thickness and hence pore length. The spread of values in Fig. 4 may be attributable to the "thicker" shells having an unusually thick surface crystal layer deposited on the column layer whilst "thinner" shells may be the result of "premature" poisoning of calcification in the shell gland or "early" oviposition compared to eggs lying along the "expected" line. The absence of sufficient observations of fine structure however makes it impossible to comment further.

As a first approximation the avian eggshell can be considered as being composed of columns of calcite (see Tullett, 1975). This simple model prognosticates some of the relationships described previously. For example, since shell thickness has been shown to increase with an increase in egg weight (Equation 4) and is inversely proportional to cones/0·25 mm^2 (Equation 10) it is not surprising that as eggs became larger and the columns became of greater diameter, that the spaces or pores between the columns also became larger (hence Equation 8). Since cone density also determines pore density (Tullett, 1975) it is not surprising that in larger eggs with columns of greater diameter the number of pores per unit area decreases (hence Equation 9).

How does a bird construct an egg which so neatly fits into this simple scheme and what happens if a change in the environmental conditions necessitates a change in eggshell porosity? The brief answer given by Tullett (1975) was based on the conclusion that the distribution of the cones in an eggshell determines the distribution of the pores. The initial spacing of the cones is determined by the seeding sites for calcification laid down on the outer surface of the outer shell membrane of the developing egg. Environmental pressures probably select for a certain eggshell porosity at the level of the cells in the isthmus of the oviduct which secrete these seeding sites. In this way the distribution of the seeding sites on which the cones develop, and hence the distribution of the pores, can be set. During shell manufacture the formation of pores is probably a simple physical consequence of the nature of calcite crystal growth, the canals between the developing columns being kept open by an ingress of liquid into the egg as described by Tyler & Simkiss (1959).

The present paper, however, presents data which suggest that a selection for a certain eggshell porosity may be achieved by a selection for a particular egg size. Thus, as an egg becomes larger (heavier) four major changes seem to occur in relation to the components of shell porosity:

(a) Less pores per cm^2,
(b) More pores per egg,
(c) Increase in the area of each pore, and
(d) Increase in shell thickness.

The graphs presented in this communication may be viewed as "adaptive scales" along which the majority of eggs have been "moved", by changes in weight, so that their pore geometry matches the demands of the embryo. An example of an adaptive adjustment in shell porosity brought about by changes in the environmental conditions has been provided by Wangensteen, Rahn, Burton & Smith (1974) who compared the eggs of domestic fowl at sea-level with those of their progeny, housed for 15 years at high altitude. The functional pore area of the eggs laid at high altitude was reduced compared to those of their sea-level counterparts, presumably as a means of restricting the increased loss of water at altitude. When we investigated traits of these eggs they appeared to have moved along the "adaptive scale" described for functional pore area by Equation (5). The eggs laid by the birds at high altitude (HA) had become smaller (40 g) than their sea-level (SL) counterparts (52 g); substitution of egg weights into Equation (5) gives a functional pore area, $((A_p)HA)$, for the high altitude eggs of 0·88 mm² and a functional pore area, $((A_p)SL)$, of 1·22 mm² for their sea-level counterparts. Thus $((A_p)HA)/((A_p)SL)$ equals 0·72. This would seem to be in good agreement with the ratio of 0·68 (Wangensteen, Rahn *et al.*, 1974) calculated from the respective conductance values, especially in view of the small sample sizes and wide variation in the conductance values of the sea-level eggs.

In relation to egg weight, shell thickness appears normal for eggs of the Common loon but the eggs have an unusually high water vapour conductance (Rahn, pers. comm.). The nests of these birds are often wet (Campbell & Ferguson-Lees, 1972) and hence the eggs are probably exposed to a high external water vapour pressure. In order to lose the seemingly normal quota of 18 % of their initial egg weight as water during incubation (Rahn & Ar, 1974) these eggs would require a high shell conductance. If the empirically determined water vapour conductance of the shell were to be achieved by increasing the egg weight according to Equation (1) it would require the construction of a 1048 g egg (almost the size of an Ostrich egg!). This is clearly an impossible task for a bird the size of a loon and the increase in the functional pore area has been achieved in other ways. Thus, during shell formation many more pores than normal, some of which have a greater cross-sectional area than normal, are formed.

A similar nesting environment is found in the Great-crested grebe (*Podiceps cristatus*) whose nests are constructed of sodden aquatic vegetation and either tethered or just left floating on water (Olney, 1974). For their weight, eggs of these birds have a high water vapour conductance (Lomholt, 1976), although the means whereby this is achieved have not been resolved. Data derived from five species suggest that the conductance of the eggshell to water vapour is adapted to the conditions of humidity in the nesting environment (Lomholt, 1976).

The concept therefore is emerging that if changes in environmental conditions necessitate a change in eggshell porosity then this may be achieved via a number of well defined changes or "scales" made possible by the mode of shell architecture. These scales can only be used however when the size of egg required to incorporate the necessary changes is within the constructional capability of the bird.

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REFERENCES

Ar, A., Paganelli, C. V., Reeves, R. B., Greene, D. G. & Rahn, H. (1974). The avian egg; water vapour conductance, shell thickness and functional pore area. *Condor* **76:** 153–158.

Board, R. G. & Tullett, S. G. (1975). The pore arrangement in the emu (*Dromaius novaehollandiae*) eggshell as shown by plastic models. *J. Microscopy* **103:** 281–284.

Campbell, B. & Ferguson-Lees, I. J. (1972). *A field guide to birds' nests.* London: Constable.

Drent, R. H. (1970). Functional aspects of incubation in the Herring gull. *Behaviour* Suppl. **17:** 1–132.

Lack, D. (1968). *Ecological adaptations for breeding in birds.* London: Methuen.

Lomholt, J. P. (1976). Relationship of weight loss to ambient humidity of birds' eggs during incubation. *J. comp. Physiol.* **105:** 189–196.

Olney, P. J. S. (1974). Classification. In *Mitchell Beazley World Atlas of Birds.* London: Mitchell Beazley.

Rahn, H. & Ar, A. (1974). The avian egg: incubation time and water loss. *Condor* **76:** 147–152.

Rahn, H., Paganelli, C. V. & Ar, A. (1974). The avian egg: air-cell gas tension, metabolism and incubation time. *Resp. Physiol.* **22:** 297–309.

Steen, J. B. (1971). *Comparative physiology of respiratory mechanisms.* London: Academic Press.

Tullett, S. G. (1975). Regulation of avian eggshell porosity. *J. Zool., Lond.* **177:** 339–348.

Tullett, S. G. & Board, R. G. (1976). Oxygen flux across the integument of the avian egg during incubation. *Br. Poult. Sci.* **17:** 441–450.

Tullett, S. G., Board, R. G., Love, G., Perrott, H. R. & Scott, V. D. (1976). Vaterite deposition during eggshell formation in the cormorant, gannet and shag, and in "shell-less" eggs of the domestic fowl. *Acta zool., Stockh.* **57:** 79–87.

Tyler, C. & Simkiss, K. (1959). A study of the eggshells of ratite birds. *Proc. zool. Soc. Lond.* **133:** 201–243.

Wangensteen, O. D., Rahn, G., Burton, R. R. & Smith, A. H. (1974). Respiratory gas exchange of high altitude adapted chick embryos. *Resp. Physiol.* **21:** 61–70.

Wangensteen, O. D., Wilson, D. & Rahn, H. (1970/71). Diffusion of gases across the shell of the hen's egg. *Resp. Physiol.* **11:** 16–30.

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MAGNESIUM DISTRIBUTION IN AVIAN EGGSHELLS

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Abstract—1. There were two peaks in the concentration of Mg^{2+} in the eggshells of 10 species of Galliformes, one in the cone layer and the other at the outer edge of the shell; there was only one peak (in the cone layer) in those of more than 60 species of 19 other orders of birds.

2. The width of the zone of the Mg^{2+}-rich inner layer was about 50% of the total shell width in small but only 10% or less in large eggs.

INTRODUCTION

The ash of the eggshell of the domestic hen contains *ca.* 0.84% $MgCO_3$ (Romanoff & Romanoff, 1949). According to these authors, the percentage of this salt in other eggshells ranges from 0.44 for the kingfisher to 1.88 for the ostrich. Brooks & Hale (1955) were probably the first to note that there was a progressive increase in the Mg^{2+} concentration from the lowest level immediately behind the cone layer to a peak in the outermost layer of the eggshells of domestic hens. This was confirmed by Itoh & Hatano (1964) who, like Brooks & Hale (1955), analysed solutions obtained by dissolving successive layers of eggshell in a mineral acid. A similar distribution of Mg^{2+} was found in quail (*Coturnix coturnix*) eggshells by Quintana & Sandoz (1978) who used electron probe analysis to examine the polished face of radial sections. This technique is ideally suited to survey work and was used in the present study to establish whether or not the distribution of Mg^{2+} in the domestic hens' eggshell is common to that in other avian species.

MATERIALS AND METHODS

Eggshells

The eggshells and their sources are listed in Table 1.

Magnesium distribution

Pieces of eggshell were embedded in plastic (Plasticraft, Turner Research Ltd., Leeds) and a radial face polished with successively finer grades of diamond paste (final grade, 1 μm). A final polish was given with gamma grade aluminium oxide as a paste in velvet supported by plate glass. The polished surface was coated *in vacuo* with gold (*ca.* 30 nm) and examined with a JEOL JXA-50A electron probe microanalyser. A Mg standard was used to calibrate the instrument before each run. The electron beam was scanned along a line across a polished radial face, beginning in the shell membranes, continuing across the tip of a cone and terminating in the plastic at the outer edge of the shell. The results were recorded on a chart moving at a known speed.

RESULTS

Two distinct patterns of Mg^{2+} concentration in avian eggshells were noted (Fig. 1). In one there were two peaks in the concentration of this element in the radial plane of the eggshell, one immediately beneath the inner (cone) surface and the other in the outermost part of the shell. With the inner surface, the Mg^{2+} peak was not confined to the cones *per se*, it occurred also in the shell immediately overlying the fused bases of cones. This pattern, noted previously in the eggshells of domestic hens (Brooks & Hale, 1955; Itoh & Hatano, 1964) and Japanese quail (Quintana & Sandoz, 1978), was common to the eggshells of 10 species of the order Galliformes and peculiar to one, the lily trotter (*Micropara capensis*), of the order Charadriiformes (Table 1). The distribution of Mg^{2+} in the domestic hens' eggshell was maintained until the 20th day in eggs that hatched. It has not been determined whether the loss of Mg^{2+} from the cone layer immediately before hatching was due to assimilation by the embryo or merely lost due to the shell being parted from its underlying membranes. There was only one peak in the concentration of Mg^{2+} in the radial plane of the eggshells of more than 60 species belonging to 19 orders of birds (Table 1). This occurred in the cone layer of the shell (Fig. 1). The wide geographical distribution of the eggs included in this survey (Table 1) is such that there would seem to be no reason for suspecting that the birds' diet *per se* was the cause of the patterns shown in Fig. 1.

A general relationship (Fig. 2) was noted when the width of the region of relatively high Mg^{2+} concentration in the cone layer (Fig. 1) was compared with the total width of the shell. The relationship obtained with eggs having either pattern A or B of Mg^{2+} distribution. In practice it was found that the inner band of Mg^{2+}-rich shell accounted for *ca.* 50% of the shell's width in the small eggs of the Passeriformes but only 10% of the thick shells of the emu and rhea.

DISCUSSION

This survey was concerned with the distribution rather than the actual concentration of Mg^{2+} in the avian eggshell. The observations pose questions about the site of Mg^{2+} deposition in the oviduct and the role of this element in the form and hence function of avian eggshells. It is now generally accepted that calcification begins in the "red region" (the distal 4 cm)

Table 1. The birds' eggs included in this survey together with the pattern of the distribution of magnesium in their shells

Pattern	Order	Species
PATTERN A*	GALLIFORMES	White browed guan, Penelope jacucaca (Z); Ring-necked pheasant, Phasianus colchicus (FS-UK); Impeyan pheasant, Lophophorus impeyanus (Z); Cheer pheasant, Catreus wallichi (Z); Quail, Coturnix coturnix (Z); Red jungle fowl, Gallus gallus (Z); Domestic hens, Gallus gallus (domestic form) (F); Guinea fowl, Numidia meleagris (F); Turkey, Meleagris gallopavo (F); Peahen, Pavo cristatus (Z).
	CHARADRIIFORMES**	Lily trotter, Micropara capensis (FS-AF).
PATTERN B*	STRUTHIONIFORMES	Ostrich, Struthio camelus (Z).
	RHEIFORMES	Rhea, Rhea americana (Z).
	CASUARIIFORMES	Emu, Dromaius novae-hollandiae (Z).
	TINAMIFORMES	Elegant crested tinamou, Eudromia elegans (F; M); Highland tinamou, Nothocercus bonapartei (M); Red-winged tinamou, Rhyncotis rufescens (M); Spotted tinamou, Nothura maculosa (M).
	GAVIIFORMES	Common Coon or Great Northern Diver, Gavia immer (FS-NA).
B	PODICIPEDIFORMES	Great-crested grebe, Podiceps cristatus (FS-UK).
	SPHENISCIFORMES	King penguin, Aptenodytes patagonia (M); Adelie penguin, Pygoscelis adeliae (FS-AN); Chinstrap penguin, Pygoscelis antartica (FS-AN); Gento penguin, Pygoscalis papua (FS-AN); Macaroni penguin, Eudyptes chrysolophus (FS-AN); Black-footed penguin, Sphenicus demursus (Z).
	PROCELLARIIFORMES	Grey-headed albatross, Diomedia chrysostoma (FS-AN); Black-browed albatross, Diomedia melanophris (FS-AN); White-chinned petrel, Procellaria aequinoctialis (FS-AN); Giant petrel, Macronectes giganteus (FS-AN); Snow petrel, Padrogoma nivea (FS-AN); Shearwater, Puffinus pacificus (FS-NA).
	PELECANIFORMES	Gannet, Sula bassana (FS-UK); Cormorant, Phalacrocorax carbo (Z).
	CICONIIFORMES	Grey heron, Ardrea cinerea (FS-UK); Black-headed heron, Ardrea melanocephala (FS-AF); White stork, Ciconia alba (FS-AF); Yellow-billed stork, Myceteria ibis (FS-AF); Chilean flamingo, Phoenicopterus chilensis (Z); Rosy flamingo, Phoenicopterus ruber (Z).

Table 1.—*cont.*

Pattern	Order	Species
	ANSERIFORMES	Merganser, Mergus cucullatus (FS-NA).
B	FALCONIFORMES	Osprey, Pandion haliactus (M); Peregrine falcon, Falco peregrinus (Z).
	GRUIFORMES	Weka rale, Gallirallus australis (FS-NA); Stanley crane, Anthropoides paradisaea (Z); Sarus crane, Grus antigone (Z); Lesser sandhill crane, Grus canadensis (FS-AN); Pukeko, Porphyrio melanotus (M).
	CHARADRIIFORMES	Whimbrel, Numenius phacopus (FS-NA); Bar-tailed godiwit, Limosa lapponica (FS-NA); Spurwing plover, Vanellus spinosa (M); Golden plover, Pluvialis apricaria (FS-NA); Western sandpiper, Calidris mauri (FS-NA); Skua, Catharacta skua (FS-NA); Mew gull, Larus canus (FS-NA); Glaucous gull, Larus glaucescens (FS-NA); Murre, Uria aalge (FS-NS); Great black-backed gull, Larinus marinus (FS-NA); Tufted puffin, Linda cirrhata (FS-NA); Razorbill, Alea torda (FS-UK); Northern phalarope, Phalaropus lobatus (FS-NA); Artic tern, Sterna paradisaea (M).
	COLUMBIFORMES	Diamond dove, Geopelia cuneata (Z).
	PSITTACIFORMES	Budgerigar, Mellopsittacus undulatus (Z).
	CUCULIFORMES	Smooth billed ani, Crotophagi ani (M); Guira cuckoo, Guira guira (M).
B	STRIGIFORMES	Eagle owl, Bubo bubo (Z).
	PASSERIFORMES	House sparrow, Passer domesticus (FS-UK); Yellow warbler, Dendroica petechia (FS-NA); Robin, Erithacus rubecula (FS-UK); Grackle, Quiscalus quiscala (FS-NA); American robin, Turdus migratorius (FS-NA); House wren, Troglodytes aedon (FS-NA); Blackbird, Turdus merula (FS-UK); Starling, Sturnus vulgaris (FS-UK).

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of the isthmus and is completed in the pouch of the shell gland of the domestic hen (Wyburn *et al.*, 1973). Initiation takes about 30 min, completion upwards of 20 hr. As the tips of the mammillae (or cones) are the first sites of calcification and as their number and distribution probably determines the porosity of the shell and its ultimate thickness (Tullett, 1975; Tullett & Board, 1977; Tyler & Fowler, 1978), this region may play an important part in determining the form and function of the shell. The question of control

mechanisms has been made topical by the observations (Rahn *et al.*, 1977) that the eggs laid by domestic hens transferred abruptly from sea level to high altitudes are soon endowed with a reduced porosity so that the water in the eggs is conserved during incubation.

The better known function of the isthmus, "plumping", may well interact with calcification to influence the final form of the eggshell. "Plumping", the absorption of water, K^+ and glucose by the albumen, causes an increase in volume whereby the enveloping shell membranes are made taut and, presumably, the nucleation sites, the tufts of fibres on their outer surface, are given a distribution that ensures pores of optimal size and a spatial relationship that provides optimal shell thickness. Although Mg^{2+} is a minor component of the secretions of the "red region"—*ca.* 1.36 mmol/l (El Jack & Lake, 1967)—the peak in its concentration in the cone layer was generally comparable to that at the outer surface of the Galliformes eggshell, the latter being laid down from solutions containing upwards of 10 mmol/l Mg^{2+}. This suggests that the cone layer has an avidity for this element greater than that of other regions of the shell. If this be so, then does Mg^{2+} play a part in ensuring that the cones retain their identity and geometry, as has been shown by electromicroscopy (Fujii, 1974; Fujii & Tamara, 1970; Creger *et al.*, 1976), until their bases fuse to form the foundation for the pallisade layer of the shell? There is perhaps another reason for invoking the concept of a Mg^{2+}-binder because of this element's inhibition of nucleation and calcite growth (Pytkowicz, 1965; Berner, 1975). Indeed this initial stage of calcification merits further investigation because of the complex processes involved. Thus "plumping" must presumably occur without calcifica-

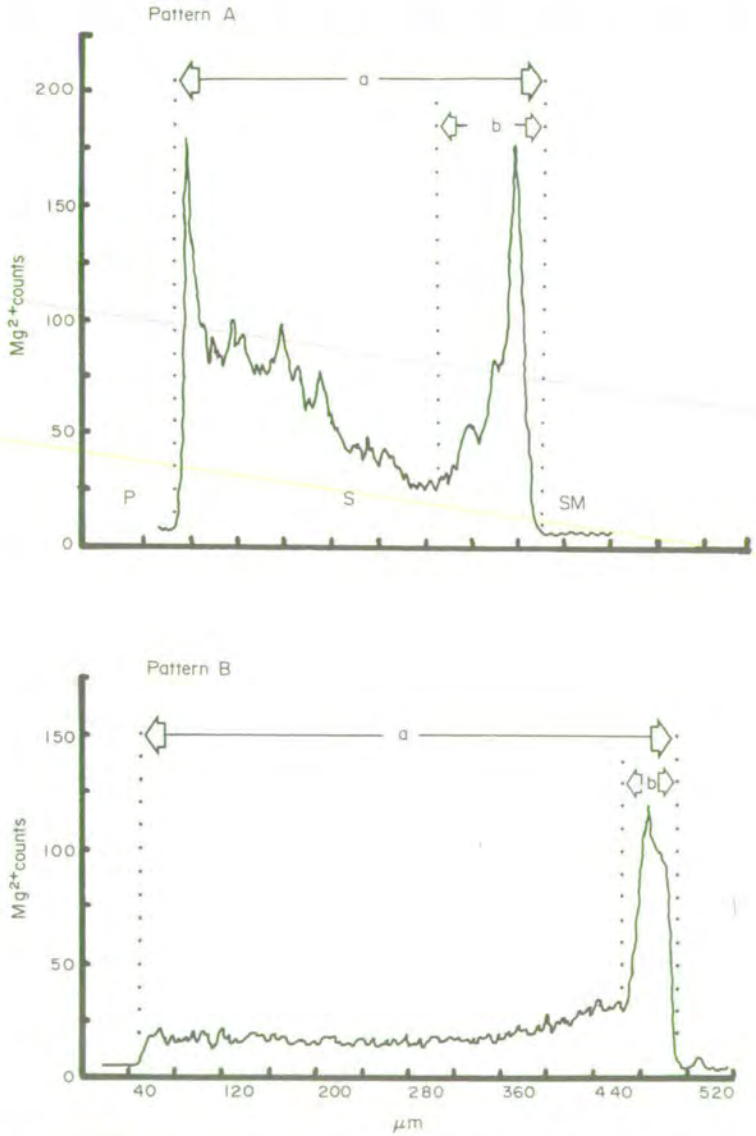

Fig. 1. The patterns obtained with electron probe analysis of polished radial sections of eggshells. The diagrams were traced from paper charts obtained with an eggshell of a domestic hen (Pattern A) and a gannet (Pattern B). SM, shell membrane; S, shell and P, plastic. The regression of a on b is shown in Fig. 2.

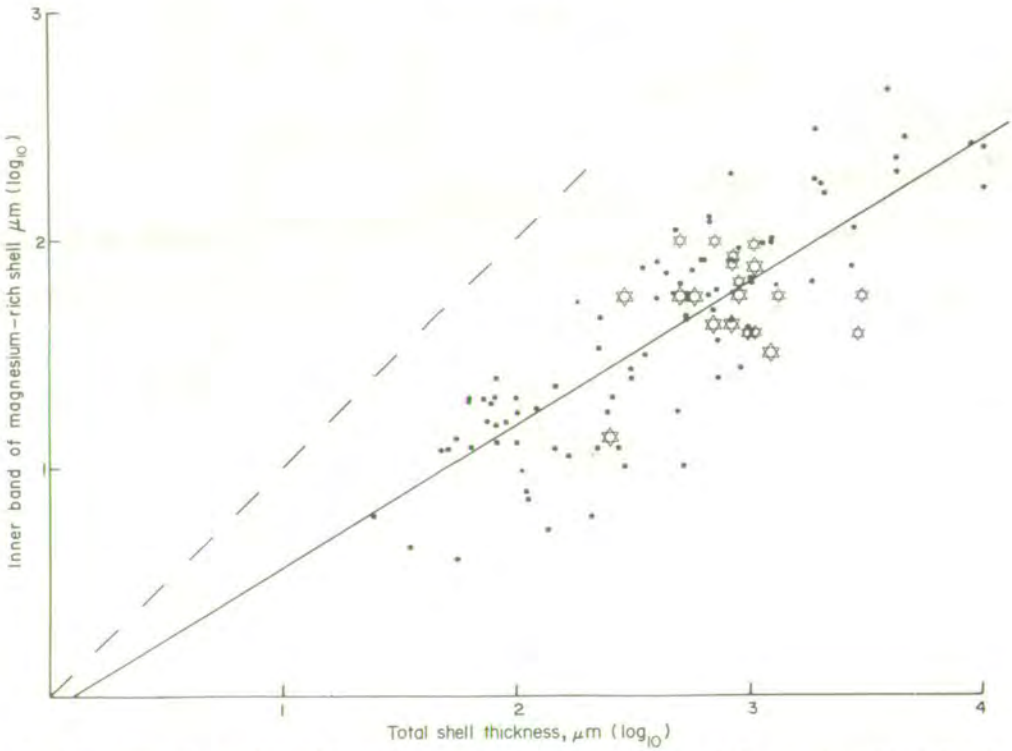

Fig. 2. The regression analysis of the width (radial plane) of the inner band of magnesium rich shell and total shell thickness (a and b of Fig. 1). Stars, shells having Pattern A and dots, Pattern B of Fig. 1. Broken line, x = y; solid line, $r^2 = 0.6189$ (r^2 = coefficient of determination).

tion of the shell membranes and yet the membranes have to be endowed with properties that permit Ca^{2+}-transport when the embryo uses the shell's calcium for bone formation. Could it be that the albumen, shell membranes and nucleation sites of the mammillae function as ion exchange systems so that the appropriate ions from the secretions of the isthmus are correctly partitioned during "plumping" and the beginnings of calcification? Moreover, the fact that Mg^{2+} lowers the solubility of calcite (Lorens & Bender, 1977) may have a bearing, as suggested by Quintana & Sandoz (1978), on the mechanism used by the embryo to obtain Ca^{2+} from the shell.

If *in vivo* studies should support our contention that Mg^{2+} secreted by the "red region" is in some way involved in determining the ultimate architecture of the shell, then the regression of the radial width of the Mg^{2+}-rich shell of the cone region against total shell thickness (Fig. 2) might be an index of the time that the egg spends under conditions that can influence the ultimate form of its shell. We assume that the pouch of the shell gland is concerned with gross calcification only. As the resident times of the egg in the "red region" and shell gland can only be determined with birds kept in captivity, our observations might permit deductions to be made about the resident times of eggs in birds in nature.

Brooks & Hale (1955) found that the hardness of the hens' eggshell was correlated with the molar Mg^{2+}:Ca^{2+} ratio, the greater the proportion of Mg^{2+} the harder the shell. A similar relationship was noted by Fox (1976) in eggs laid by Common Tern receiving a diet contaminated with DDE. This suggests that this compound is influencing the Mg^{2+} secretion of the oviduct with the result that an increased hardness (and thus brittleness) makes the shell more vulnerable to denting on impact with other objects in the nest.

SUMMARY

Electron probe microanalysis was used to survey the distribution of Mg^{2+} in the radial axis of the eggshells of birds belonging to 20 orders of birds. All the eggshells had a narrow band of Mg^{2+}-rich shell in the cone layer. With the exception of the lily trotter (*Micropara capensis*), a progressive increase in the concentration of Mg^{2+} from the lowest level immediately beneath the cone layer to a peak at the surface was a characteristic feature of the eggshells of members of the Galliformes.

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REFERENCES

Berner R. A. (1975) The role of magnesium in crystal growth of calcite and aragonite from sea water. *Geochim. Cosmochim. Acta* **39**, 489–504.

Brooks J. & Hale H. P. (1955) Strength of the shell of the hen's egg. *Nature, Lond.*, **175**, 848–849.

Creger C. R., Phillips H. & Scott J. T. (1976) Formation of an eggshell. *Poult. Sci.* **55**, 1717–1723.

El Jack M. H. & Lake P. E. (1967) The content of the

principal inorganic ions and carbon dioxide in uterine fluids of the domestic hen. *J. Reprod. Fert.* **13,** 127–132.

FOX G. A. (1976). Eggshell quality: its ecological and physiological significance in a DDE-contaminated common tern population. *The Wilson Bulletin* **88,** 459–477.

FUJII S. (1974) Further morphological studies on the formation and structure of hen's eggshells by scanning electron microscopy. *J. Fac. Fish Anim. Husb. Hiroshima Univ.* **13,** 29–56.

FUJII S. & TAMARA T. (1970) Scanning electron microscopy of shell formation in hen's eggs. *J. Fac. Fish Anim. Husb. Hiroshima Univ.* **9,** 65–81.

ITOH H. & HATANO T. (1964) Variation of magnesium and phosphorous deposition rates during eggshell formation. *Poult. Sci.* **43,** 77–80.

LORENS R. B. & BENDER M. B. (1977) Physiological exclusion of magnesium from *Mytilus edulis* calcite. *Nature, Lond.* **269,** 793–794.

PYTKOWICZ R. M. (1965) Rates of inorganic calcium carbonate nucleation. *J. Geol.* **73,** 196–199.

QUINTANA C. & SANDOZ D. (1978) Coquille de l'oeuf de caille: étude ultrastructurale et cristallographique. *Calcif. Tiss. Res.* **25,** 145–159.

RAHN H., CAREY C., BALMAS K., BHATA B. & PAGANELLI C. (1977) Reduction of the pores of the avian eggshell as an adaptation to altitude. *Proc. natn. Acad. Sci. U.S.A.* **74,** 3095–3098.

ROMANOFF A. L. & ROMANOFF A. J. (1949) *The Avian Egg.* Wiley, New York.

TULLETT S. G. (1975) Regulation of avian eggshell porosity. *J. Zool., Lond.* **177,** 339–348.

TULLETT S. G. & BOARD R. G. (1977) Determinants of avian eggshell porosity. *J. Zool., Lond.* **183,** 203–211.

TYLER C. & FOWLER S. (1978) The distribution of organic cores, cones, cone junctions and pores in the eggshells of wild birds. *J. Zool., Lond.* **186,** 1–14.

WYBURN G. M., JOHNSTON H. S., DRAPER M. H. & DAVIDSON M. F. (1973) The ultrastructure of the shell forming region of the oviduct and the development of the shell of *Gallus domesticus*. *Q. J. exp. Physiol.* **58,** 143–151.

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MAGNESIUM DISTRIBUTION IN AVIAN EGGSHELLS WITH PARTICULAR REFERENCE TO THOSE OF WILDFOWL (ANATIDAE)

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Abstract—1. There were two major peaks in the concentration of Mg^{2+} in the eggshells of 3 species of Galliformes, 2 species of Pteroclidiformes and 35 species of Anseriformes; one peak occurred in the cone layer and the other at the outer edge of the shell.

2. There was only one major peak (in the cone layer) in the eggshells of 99 species of birds belonging to the Megapodidae (Galliformes) or 12 orders of birds other than those listed above.

INTRODUCTION

Although it has been known for a long time (Romanoff & Romanoff, 1949) that there are appreciable differences in the magnesium content of avian eggshells—0.44% $MgCO_3$ in the Kingfisher and 1.88% in the Ostrich eggshell—profound differences in the distribution of this element in the radial plane of shells were not recorded until 1980 (Board & Love). Their survey with an electron probe microanalyser revealed two peaks in the Mg^{2+} concentration in the eggshells of members of the Galliformes, one peak occurring in the cone layer and the other at the outer edge of the shell. Only one peak (in the cone layer) was noted in the eggshells of birds belonging to 19 other orders of birds. One exception only was noted. The eggshells of the Lily Trotter (*Micropara capensis*) differed from other members of the order Charadriiformes in having twin peaks in the Mg^{2+} concentration. Indeed the pattern of distribution in the eggshells of this species resembled that which was characteristic of those of the Galliformes. This survey has been extended and particular attention given to eggshells of wildfowl (tribe Anatidae). The results presented in this report suggest that the differences in the pattern of Mg^{2+} distribution across avian eggshells reflect important differences in the physiology of the shell gland and/or mineral metabolism in different orders of birds and that these differences may have phylogenetic implications.

MATERIALS AND METHODS

Eggshells

The eggshells and their sources are listed in Tables 1 and 2.

Magnesium distribution

Pieces of eggshell were embedded in plastic (Plasticraft, Turner Research Ltd., Leeds) and a radial face polished with successively finer grades of diamond paste (final paste grade, 1.0 μm). The polished face was coated *in vacuo* with gold (*ca* 30 nm) and examined with a JEOL JXA-733 electron probe microanalyser. The electron beam was scanned along a line across a polished radial plane, beginning in the shell membranes, continuing across the tip of a cone and terminating in the outer edge of the shell. The results were recorded on paper chart moving at known speed.

RESULTS AND DISCUSSION

The electron probe microanalyser (JEOL JXA-733) used in this study was more sensitive than that (JEOL JXA-53A) employed previously (Board & Love, 1980). This may well be the reason for our recognition of a third pattern of Mg^{2+} distribution across the radial plane of eggshells, A_v (v standing for vestigial) in Fig. 1. It was characterized by a major peak in the cone layer and a minor one in the outermost portion of the shell. The latter peak was small in height when compared to that found in eggshells having Pattern A for Mg^{2+} distribution. Moreover the origin of this minor peak in A_v eggshells tended to be nearer the outer edge rather than the midpoint of a shell. With those having the A Pattern (see the trace for domestic hens' eggshells in Fig. 1), the origin of the second (outermost) peak was coincident with the lowest count for Mg^{2+} following the peak in the cone layer. In other words, there was no discernible flat portion between the two peaks.

For the purposes of a broad categorization of eggshells, those having Pattern A_v have been included in Pattern B in Table 1. If the results obtained with wildfowl eggs (Table 2) are set aside for the moment, then the results summarized in Table 1 confirm our earlier observations (Board & Love, 1980), namely that Pattern B is a common feature to eggshells of birds belonging to orders other than Galliformes. Indeed this situation is accentuated when a summary (Table 3) of the results of our two surveys is considered without attention being given to the Anseriformes. The few exceptions to the general pattern were found with birds whose classification is problematical or whose methods of incubation are unusual.

The Namaqua and Double-banded sandgrouse lay eggs with Pattern A Mg^{2+} distribution in the shells.

Table 1. The birds' eggs included in this survey together with the pattern of magnesium in their shells

Pattern	Order (Family)	Species
B	Apterygiformes	Kiwi, *Apteryx* sp. (T*)
B	Podicipediformes	Pied-billed grebe, *Podilymbus podiceps* (RA-US)
B	Procellariiformes	Wedge-tailed shearwater, *Puffinus pacificus* (RA-US)
		Muttonbird, *Puffinus tenuirostris* (T)
		Fairy prion, *Pachyptila turtur* (RG-An)
	Pelecaniformes	Tropic bird, *Phaethon* sp. (PH-Maur)
	Ciconiiformes	Scarlet ibis, *Eudocimus ruber* (Z)
A/B	Anseriformes	See Table 2
B	Falconiformes	Hobby, *Falco subbuteo* (T)
		Secretary bird, *Saggitarius serpentarius* (T)
		Buzzard, *Buteo buteo* (T)
		Kestrel, *Falco* sp. (T)
B	Galliformes	Scrub fowl, *Megapodius freycinet* (RS-Aus)
	(Megapodidae)	Scrub hen, *Megapodius* sp. (M)
		Mallee fowl, *Leipoa ocellata* (RS-Aus)
		Brush turkey, *Alectura lathami* (RS-Aus)
A	(Phasianidae)	Red-legged partridge, *Alectoris rufa* (T)
		Domestic hen, *Gallus domesticus* (F)
		Willow ptarmigan, *Lagopus lagopus* (T)
B	Gruiformes	Black bellied bustard, *Lissotis melanogaster* (Z)
		Stanley's crane, *Anthropoides paradisea* (Z)
		Crowned crane, *Balearica pavonina* (Z)
		Demoiselle crane, *Anthropoides virgo* (Z)
B	Charadriiformes	Oyster catcher, *Haemotopus ostralegus* (F)
A	Columbiformes	Namaqua sandgrouse, *Pterocles namaqua* (DT-SA)
	(Pteroclididae)	Double-banded Sandgrouse, *Pterocles bicinctus* (DT-SA)
B	(Columbidae)	Pink pigeon, *Columba mayeri* (PH-Maur)
B	Psittaciformes	Yellow-collared lovebird, *Agapornis personata* (GB-US)
		Peach-faced lovebird, *Agapornis roseicollis* (GB–US)
		Cockatiel, *Nymphicus hollandicus* (GB–US)
B	Strigiformes	Great-horned owl, *Bubo virginianus* (Z)
		Woodford's owl, *Ciccalba woodfordii*
		Barn owl, *Tyto alba* (Z)
		Kazaki owl, *Bubo bubo omissus* (Z)
		Malaysian fish owl, *Ketupa ketupu* (Z)
		Indian eagle-owl, *Bubo bubo bengalensis* (Z)
B	Passeriformes	Grey shrike, *Lanius* sp. (T)
		Mistle thrush, *Turdus viscivorus* (T)
		Greenfinch, *Carduelis chloris* (T)
		Larks: Alaudidae (T)
		Corn bunting, *Emberiza callandra* (T)
		Magpie, *Pica pica* (T)
		Hooded crow, *Corvus* sp. (T)
		Rook, *Corvus frugilegus* (T)
		Jackdaw, *Corvus monedula* (T)
		Carrion crow, *Corvus corone* (T)
		Blackbird, *Turdus merula* (F)
		Starlings: Sturnidae (F)

* Origin of eggs.

T, the collection of Professor C. Tyler; RA-US, Dr Ralph Ackerman, USA; Z, Mr P. J. Olney, London Zoo; RS-AUS, Dr Roger Seymour, Australia; M, Museum; F, farm or field; DT-SA, David Thomas, South Africa; GB-US, Professor George Bartholomew, USA; PH-Maur, Paul Howey, Mauritius, RG-An, Dr R. Grau, Antarctica.

Although this pattern is characteristic of the Galliformes, these grouse are variously classified as a family (Pteroclididae) in the order Charadriiformes (Thomas & Maclean, 1981) or the order Columbiformes (Howard & Moore, 1980) or as an order (Pteroclidiformes) in their own right (viz. Mr P. J. S. Olney in Bramwell, 1974). It is noteworthy that another member, the Lily-Trotter (*Micropara capensis*), of the Charadriiformes lays eggs with Pattern A Mg^{2+} distribution.

Eggshells of members of the family Megapodidae of the order Galliformes provided another exception to the general trends evident in Tables 1 and 3. Birds belonging to this family are notable for the practice of incubating their eggs in mounds of sand/soil/vegetable matter heated by insolation, microbial fermentation or geothermal energy. It has been found that the conductance of their eggshells is adapted such that they allow the embryo to exchange respiratory gases with the CO_2-rich but O_2-depleted atmosphere of the

Table 2. The wildfowl eggs included in this study and the Mg^{2+} pattern in their eggshells*

	Mg^{2+} pattern		
	A	A_v†	B
Family Anatidae			
Sub-family Anseranatinae			
Tribe Anseranatini			
Magpie Goose *Anseranas semipalmata*	+		
Sub-family Anserinae			
Tribe Dendrocygnini			
Spotted Whistling Duck *Dendrocygna guttata*	+		
Fulvous Whistling Duck *Dendrocygna bicolor*	+		
Cuban Whistling Duck *Dendrocygna arborea*	+		
White-faced Whistling Duck *Dendrocygna viduata*	+		
Southern Red-billed Whistling Duck *Dendrocygna autumnalis discolor*	+		
Tribe Anserini			
Coscoroba Swan *Coscoroba coscoroba*	+		
Black Swan *Cygnus atratus*		+	
Mute Swan *Cygnus olor*		+	
Black-necked Swan *Cygnus melanocoryphus*		+	
Whooper Swan *Cygnus cygnus cygnus*			+
Trumpeter Swan *Cygnus cygnus buccinator*		+	
Bewick's Swan *Cygnus columbianus bewickii*			+
Moffitt's Canada Goose *Branta canadensis moffitti*	+		
Domestic Goose	+		
Swan Goose *Anser cygnoides*	+		
Bean Goose *Anser fabalis*	+		
Lesser White Fronted Goose *Anser erythropus*	+		
Pacific White Fronted Goose *Anser albifrons frontalis*	+		
Eastern Greylag Goose *Anser anser rubrirostris*	+		
Greenland White Fronted Goose	+		
Barheaded Goose *Anser indicus*	+		
Emperor Goose *Anser canagicus*	+		
Lesser Snow Goose *Anser coerulescens coerulescens*	+		
Ross's Goose *Anser rossii*		+?	
Giant Canada Goose *Branta canadensis maxima*	+		
Dusky Canada Goose *Branta canadensis occidentalis*	+		
Aleutian Canada Goose *Branta canadensis leucopareia*	+		
Cackling Canada Goose *Branta canadensis minima*	+		
Ne-ne *Branta sandvicensis*	+		
Barnacle Goose *Branta leucopsis*	+		
Great Snow Goose *Anser coerulescens atlanticus*	+		
Red-breasted Goose *Branta ruficollis*			
Lesser Canada Goose *Branta canadensis parvipes*	+		
Sub-family Anatinae			
Tribe Tadornini			
Cereopsis Goose *Cereopsis novae-hollandiae*	+		
Lesser Magellan Goose *Chloëphaga picta picta*		±	
Cape Shelduck *Tadorna cana*		±	
Paradise Shelduck *Tadorna variegata*		±	
Radjah Shelduck *Tandorna radjah radjah*		±	
Patagonian Crested Duck *Lophonetta specularioides specularioides*		+	
Ruddy Shelduck *Tadorna ferruginea*		±	
Andean Crested Duck *Lophonetta specularioides alticola*		±	
Tribe Anatini			
Marbled Teal *Marmaronetta angustirostris*		+	
Bronze-winged Duck *Anas specularis*		±	
Chilean Pintail *Anas georgica spinicauda*		±	
Australian Grey Teal *Anas gibberifrons gracilis*			+
New Zealand Brown Duck		+	
Mallard *Anas platyrhynchos platyrhynchos*		+	
Domestic Duck		±	
Mexican Duck *Anas platyrhynchos diazi*		+	
Indian Spotbill *Anas poecilorhyncha poecilorhyncha*			+
Pelew Island Grey Duck *Anas superciliosa pelewensis*			+
Philippine Duck *Anas luzonica*		+	
Meller's Duck *Anas melleri*			+
Chinese Spotbill *Anas poecilorhyncha zonorhyncha*			+
European Wigeon *Anas penelope*			+
Argentine Cinnamon Teal *Anas cyanoptera cyanoptera*			+
Northern Cinnamon Teal *Anas cyanoptera orinomus*		±	

(continued)

Table 2 (*continued*).

	Mg^{2+} pattern		
	A	A_v†	B
Pink-eared Duck *Malacorhynchus membranceus*		±	
Yellowbill *Anas undulata*			+
Ringed Teal *Calonetta leucophrys*			+
Tribe Somateriini			
Spectacled Eider *Somateria fischeri*			+
Northern Eider *Somateria mollissima borealis*		+	
European Eider *Somateria mollissima mollissima*		+	
Tribe Aythyini			
Southern Pochard *Netta erythrophthalma erythrophthalma*		±	
Lesser Scaup *Aythya affinis*		+	
New Zealand Scaup *Aythya novae-hollandiae*		+	
Baer's Pochard *Aythya baeri*		+	
Tribe Cairinini			
Muscovy Duck *Cairina moschata*			+
Mandarin Duck *Aix galericulata*			+
Carolina Duck *Aix sponsa*			+
White-winged Wood Duck *Cairina scutulata*			+
Tribe Mergini			
European Goldeneye *Bucephala clangula clangula*			+
Barrow's Goldeneye *Bucephala islandica*		+	
Hooded Merganser *Mergus cucullatus*		±	
Smew *Mergus albellus*		+	
Scoter *Melanitta* sp.			+
Harlequin Duck *Histrionicus histrionicus histrionicus*		+	
Tribe Oxyurini			
North American Ruddy Duck *Oxyura jamaicensis jamaicensis*	+		
Africa White-backed Duck *Thalassornis leuconotus leuconotus*	+		
Black-headed Duck *Heteronetta atricapilla*	+		
White-headed Duck *Oxyura leucocephala*	+		

* The eggs were taken from the collections of Professor C. Tyler, Dr Gill Bond and Nick French. The White-Backed Duck eggshell was kindly donated by Professor G. V. T. Matthews.
† Pattern pronounced, ±; slight, +, or very slight, ±.

mound (Seymour & Ackerman, 1980; Board *et al.*, 1982). As significant thinning (the shells are *ca* 2.5 times thinner than those on eggs of comparable mass laid by other birds) is one adaptation that enhances conductance, the single peak in the Mg^{2+} distribution in megapode eggshells may provide a clue to the mechanisms which result in a diminution of shell width. It was noted previously (Board & Love, 1980) that the width of the Mg^{2+}-rich inner layer was about 50% of the total shell width in small (thin shelled) eggs but only 10% or less in large eggs having shell thickness appropriate to mass. This trend, which was an obvious feature of the parent survey, is evident in Fig. 1. Board & Love (1980) surmised that the width of the Mg^{2+}-rich band in the cone layer relative to total shell thickness may provide an index of the duration of two events in shell formation, cone formation which probably occurs in the distal portion of the isthmus (Wyburn *et al.*, 1973) and which determines shell porosity (Tyler & Fowler, 1978; Tullett, 1978), and bulk mineralization in the shell gland. It would seem reasonable to assume that megapode eggshells are thin and contain only one peak in the concentration of Mg^{2+} because of a very brief stay in the shell gland.

All three patterns (Fig. 1) of Mg^{2+} distribution across the radial plane were found in the eggs laid by members of the family Anatidae (Table 2) which were examined with the new electron probe microanalyser (JEOL JXA-733). Pattern A (Fig. 1) was found with the eggshells of the Magpie goose (*Anseranas semipalmata*) of the sub-family Anserenatinae and the 5 species of Whistling or Tree Ducks (tribe Dendrocygnini) of the subfamily Anserinae. Another tribe, Anserini, of this latter subfamily contained eggs having Patterns A, A_v or B for Mg^{2+} distribution. Pattern A was characteristic of geese eggshells whereas, with the exception of the Coscobora swan (*Coscobora coscobora*), Patterns A_v and B were common to eggshells of swans. With the third sub-family, Anatinae, Patterns A_v or B were found in the eggshells of 45 species of the tribes Tadornini (Shelducks and Sheldgeese), Anatini (Dubbling ducks), Somateriini (Eiders), Aythyini (Pochards), Cairinini (Wood ducks etc.) and Mergini (Scoters etc.). The only exception was provided by eggshells (Pattern A) of the Cereopsis goose (*Cereopsis novaehollandiae*) which is listed by Scott (1968) as an aberrant species with affinities to the Tribe Tadornini. It was notable, however, that the most exaggerated form of the A_v Pattern (Table 2) was found with members of this tribe, viz. the Cape, Paradise, Radjah and Ruddy Shelducks. With the remaining species of this tribe and those of the other 4 tribes listed above, the Patterns A_v (slight or very slight) and B occurred at about equal frequency.

Judging from the review by Johnsgard (1966), taxonomic problems abound in the tribe Oxyurini. He proposed reclassification of the White-backed duck (*Thalassornis leuconotus leuconotus*)—behavioural and anatomical features warranted its classification in the

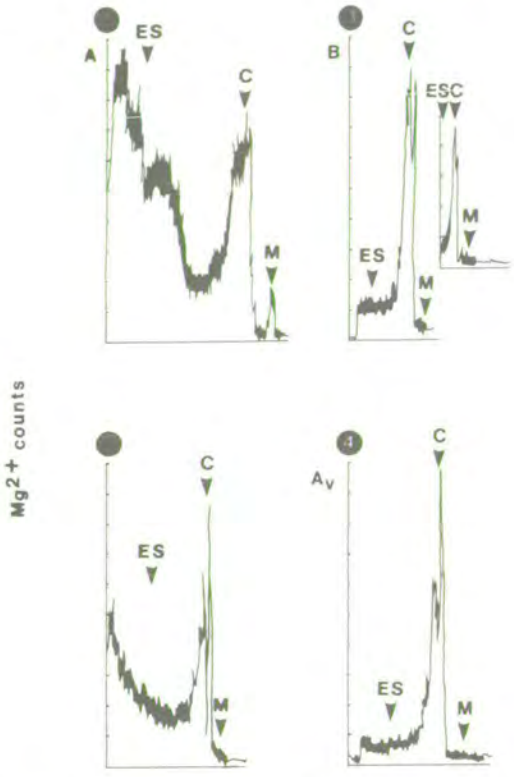

Fig. 1. Mg^{2+} distribution across the radial axis of avian eggshells. (1) The A Pattern typical of domestic hens and related birds; (2) the A Pattern typical of the eggshells of Geese; (3) the B Pattern (insert, a trace obtained with the eggshell of a member of the Passeriformes), and (4) the A_v Pattern obtained with ducks—a small increase in the Mg^{2+} counts is evident in the outermost portion of the shell. M, shell membranes; C, conelayer; ES, eggshell proper.

tribe Dendrocygnini; suggested that the Black-headed duck (*Heteronetta atricapilla*) was the "most generalized of the true stiff-tails" (tribe Oxyurini), and concluded that species of the genus *Oxyura* fall into two broad evolutionary groups. In the light of these observations, it is notable that we found Pattern A for Mg^{2+} distribution across the radial plane of the shells of the 4 species (Table 2) of the tribe Oxyurini included in this study. Thus with the exception noted above—the Cereopsis goose—the eggshells of these four species (American Ruddy Duck, White-backed Duck, Black-headed Duck and White-headed Duck) are more akin to those of members of the sub-families Anseranatinae and Anserinae than they are to the sub-family, Anatinae, to which they are now assigned.

Although our survey has confirmed and extended the observations of an earlier one (Board & Love, 1980) and drawn attention to possible phylogenetic implications of the pattern of Mg^{2+} distribution across the radial plane of the shell, it is not possible to offer an explanation of the three Patterns A, A_v and B (Fig. 1) in terms of the physiology of shell formation. This is due to the limited amount of work done on shell calcification and the overwhelming emphasis that has been given to this process in the shell gland of the domestic hen (Mongin & Carter, 1977).

Table 3. A summary of the patterns of magnesium distribution in the eggshells of the birds of twenty orders of birds*

Order (family)	Pattern A	Pattern B†
Apterygiformes		1+
Struthioniformes		1
Rheiformes		1
Casuariiformes		1
Tinamiformes		4
Gaviiformes		1
Podicipediformes		2
Sphenisciformes		6
Procellariiformes		7
Pelacaniformes		2
Ciconiformes		6
Anseriformes	30	52
Falconiformes		6
Galliformes		
(Megopodidae)		4
(Tetraonidae)	1	
(Phasianidae)	9	
(Numididae)	1	
(Meleagrididae)	1	
Gruiformes		8
Charadriiformes	1*	16
Pteroclidiformes	2	
Columbiformes		2
Psittaciformes		4
Cuculiformes		2
Strigiformes		7
Passeriformes		17
Totals	45	147

* The results of the original survey (Board & Love, 1980) are included; +, number of species.

† This includes A_v (see Fig. 1).

SUMMARY

Electron probe microanalysis was used to survey the distribution of Mg^{2+} in the radial axis of birds belonging to 14 orders of birds. All the eggshells had a narrow band of Mg^{2+}-rich shell in the cone layer. In some shells, there was a progressive increase in the concentration of Mg^{2+} from the lowest level immediately below the cone layer to a peak at the outer surface of the shell. This situation obtained with the eggshell of members of the orders Galliformes other than Megapodidae and Pteroclidiformes. It was found also in the eggshells of the Magpie Goose (*Anseranas semipalmata*) and the geese but not the swans of the sub-family Anserinae of the order Anseriformes. With the sub-family Anatinae of this order, only the eggshells of four species of the tribe Oxyurini had Pattern A for Mg^{2+} distribution.

Acknowledgements—We are grateful to Dr V. D. Scott for extensive use of his facilities, the many persons (listed in Tables 1 and 2) who supplied eggshells and The Science and Engineering Research Council of the United Kingdom for the provision of the electron probe microanalyser.

REFERENCES

Board R. G. & Love G. (1980) Magnesium distribution in avian eggshells. *Comp. Biochem. Physiol.* **66A**, 667–672.

Board R. G., Perrott H. R., Love G. & Seymour R. S. (1982) A novel pore system in the eggshells of the Mallee Fowl, *Leipoa ocellata*. *J. exp. Zool.* **220,** 131–134.

Bramwell M. (1974) *The Mitchell Beazley World Atlas of Birds*. Mitchell Beazley, London.

Howard R. & Moore A. (1980) *A Complete Checklist of the Birds of the World*. Oxford University Press, Oxford.

Johnsgard P. A. (1966) Observations on the behaviour and relationships of the White-backed Duck and the Stiff-tailed Ducks. *Wildfowl* **18,** 98–107.

Mongin P. & Carter N. H. (1977) Studies on the avian shell gland during egg formation: aqueous and electrolytic composition of the mucosa. *Br. Poult. Sci.* **18,** 391–351.

Romanoff L. A. & Romanoff A. J. (1949) *The Avian Egg*. Wiley, New York.

Scott P. (1968) *A Coloured Key to the Wildfowl of the World*. The Wildfowl Trust, Slimbridge, UK.

Seymour R. S. & Ackerman R. A. (1980) Adaptations to underground nesting in birds and reptiles. *Am. Zool.* **20,** 437–447.

Thomas D. H. & Maclean G. C. (1981) Comparison of physiological and behavioural thermoregulation and osmoregulation in two sympatric sand grouse species (Aves: Pteroclididae) *J. Arid Environs.* **4,** 335–358.

Tullett S. G. (1978) Pore size versus pore number in avian eggshells. In *Respiratory Function in Birds, Adult and Embryonic* (Edited by Piiper J.), pp. 217–226. Springer, Berlin.

Tyler C. & Fowler S. (1978) The distribution of organic cores, cones, cone junctions and pores in the eggshells of wild birds. *J. Zool., Lond.* **186,** 1–14.

Wyburn G. M., Johnston H. S., Draper M. H. & Davidson M. F. (1973) The ultrastructure of the shell-forming region of the oviduct and the development of the shell of *Gallus domesticus*. *Quart. J. exp. Physiol.* **58,** 143–151.

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Crystal Orientation in the Shell of the Domestic Fowl: An Electron Diffraction Study

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Summary. The eggshell of the domestic fowl has been studied by transmission electron microscopy and diffraction. Thin sections of shell were prepared by chemical and ion-beam thinning techniques. Each calcite column of the palisade layer consisted of crystallites of diameter 20 to 30 μm with some tendency for crystallite alignment within a single column. Evidence indicates that there was no significant preferred orientation in the palisade layer as a whole. Only in the surface layer was any preferred orientation detected, and here $\{10\bar{1}4\}$ planes tended to lie parallel to the surface. The results are compared with previously published data, and calcite nucleation and growth are discussed.

Key words: Avian eggshell — Microstructure — Electron microscopy — Electron diffraction — Calcite growth.

The eggshell structure of the domestic fowl *(Gallus domesticus)* can be divided into five separate layers as shown schematically in Fig. 1. The innermost layer consists of two fibrous membranes of protein/polysaccharide approximately 70 μm thick. Crystals of calcite extend from a number of regions on the membrane surface to form a dome-like structure ~ 100 μm thick, referred to as the basal cap and cone layer. When the cones impinge on one another during calcite growth, columnar crystals develop 50 to 100 μm in diameter and ~ 200 μm in length, termed the palisade layer. The columnar crystals are covered with a thin layer ~ 8 μm thick made of small calcite crystals with their greatest dimension perpendicular to the surface [1]. The outermost regions of the shell consist of an organic cuticle typically ~ 10 μm thickness. Not shown in the diagram are the pore canals and the glycoprotein organic matrix which permeates the calcite as randomly oriented fibers ~ 0.01 μm thick and up to ~ 10 μm long [2].

A number of factors can affect the structure and growth of the crystalline shell and, in turn, the shell's integrity and its contribution to the developing embryo. As an aid to identifying these factors, investigations have been made into the morphology and characteristics of the calcite crystals. Most studies of the cone layer indicate that the crystals show no preferred orientation, but there appears to be a considerable difference of opinion concerning growth directions in the palisade layer. Terepka [3] and Schmidt [4] concluded from polarized light examinations that the crystal columns of the palisade layer of the domestic fowl had the c-axis of the hexagonal unit cell [5] perpendicular to the shell surface. In contrast, Cain and Heyn [6] deduced from X-ray diffraction studies that the c-axis of the columns was inclined at angles between 12° and 44° to the surface normal. A more limited X-ray diffraction study carried out by Favejee et al. [7] gave no data on the palisade layer but indicated the presence of a preferred orientation in the surface layers such that $\{10\bar{1}4\}$ planes were aligned parallel to the surface.

There appear to be few reported transmission electron microscopy and diffraction studies of the crystalline shell of the domestic fowl apart from some replica examinations [8] in which it was deduced that the crystals are distinctly oriented with one of the crystallographic axes parallel to the shell surface. However, thin sections have been investigated which were obtained by ultramicrotomy from the goose egg [9] and the Japanese quail [10]. The former authors gave few data on possible orientation in the calcite columns, but the latter gave some results showing the c-axis of calcite inclined at 45° and 60° to the shell normal.

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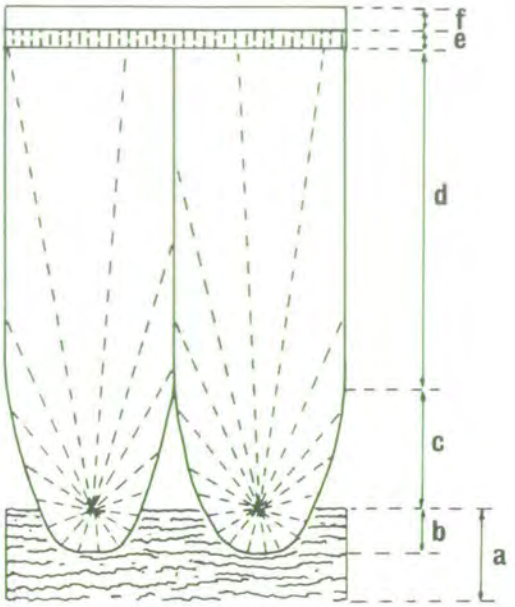

Fig. 1. Schematic diagram of radial section of eggshell: *(a)* membranes, *(b)* basal cap, *(c)* cone layer, *(d)* palisade layer, *(e)* surface crystal layer, and *(f)* cuticle

In view of the apparent difference of opinion concerning the growth directions in the palisade layer, it was decided to carry out a further study. Transmission electron diffraction was chosen since, provided thin sections could be prepared, it would be possible to examine calcite layers taken from different parts of the shell. It was also intended to investigate the technique of ion-beam thinning for specimen preparation because it should not suffer from the problems and artifacts associated with either chemical or mechanical thinning techniques.

Materials and Methods

Samples approximately 5 mm across were broken from the eggshell of the domestic hen and heated (80°C) in concentrated KOH solution to remove the shell membranes and the organic cuticle. The shell was thinned for transmission electron microscopy and diffraction using two different methods. Initially a chemical thinning technique was used and later, when suitable equipment became available, an ion-beam thinning technique was employed.

Chemical thinning was carried out in 5% HNO_3 solution at room temperature. When the first perforation of the shell occurred (after ~ 2 h), the specimen was removed from the solution and washed in deionized water. This was followed by a second immersion in hot KOH solution to remove any organic matrix and washing again in water. Thin sections from the outer surface layer of the shell were prepared in a similar way although firstly an acid-resistant lacquer (Lacomit) was applied to the outer surface so that chemical dissolution was confined to the inner layers; a solvent was then used to dissolve the lacquer prior to final treatment in hot KOH solution.

Ion-beam thinning of shells was carried out using an Edwards type E306 coating unit fitted with an ion-beam thinning attachment type 1BT200. The pieces of eggshell were clamped carefully between two metal discs containing a central hole. After the work chamber was evacuated, argon gas was passed to diametrically opposed ion guns to produce an ion beam current ~ 40 μA at 5 kV. The specimen was rotated during bombardment, and when the first perforation of the shell occurred, thinning was stopped and the specimen removed. Typical thinning time was ~ 150 h, corresponding to the removal of ~ 2 μm of material per hour. Thin specimens were first examined in a optical microscope using polarized light.

Electron microscopy and diffraction was carried out in a JEOL 100CX instrument operating at 100 kV. The chemically thinned specimens were placed within 3 mm diameter electron microscope double grids. Ion-beam thinned specimens were fitted directly in a beryllium specimen holder. All specimens were coated with a thin (< 10 nm) layer of gold to prevent charging of the specimen during examination in the microscope. The gold layer provided also a means of calibrating the electron diffraction pattern. Crystal structure and orientation of the specimens was deduced from diffraction patterns taken from selected areas < 1 μm in diameter. In order to facilitate analysis of the patterns, a stereographic projection on the (0001) plane of the calcite lattice was constructed; this diagram also served to collate data from a number of analyses.

Results

Chemically Thinned Samples

It was noted that the specimens were uneven in thickness and showed little microstructural detail when viewed in the polarizing light microscope and the transmission electron microscope. Areas < 1 μm in size were present, which were suitably thin to give single-crystal electron diffraction patterns. A typical selected area diffraction pattern is illustrated in Fig. 2A. This shows a series of spots from single-crystal calcite together with diffraction rings from the gold film on the surface. The analysis of the calcite diffraction pattern is given in Fig. 2B. From the (0001) stereogram the c-axis of the calcite crystal was determined to be at 23° to the specimen surface; its azimuthal position is indicated by the arrow in Fig. 2B, i.e., the arrow represents the projection of the c-axis in the plane of the diagram.

Several areas on each of 8 specimens consisting of sections through the palisade layer were analyzed to give 24 results. These data are shown in Fig. 3 on a (0001) stereographic projection, each circle representing the perpendicular or pole of an area. If there were any preferred orientation in the specimen, it would show as a clustering of circles about the preferred crystallographic direction. However, Fig. 3 indicates that the orientation of the different crystals is essentially random. This may be more readily appreciated by constructing an arc

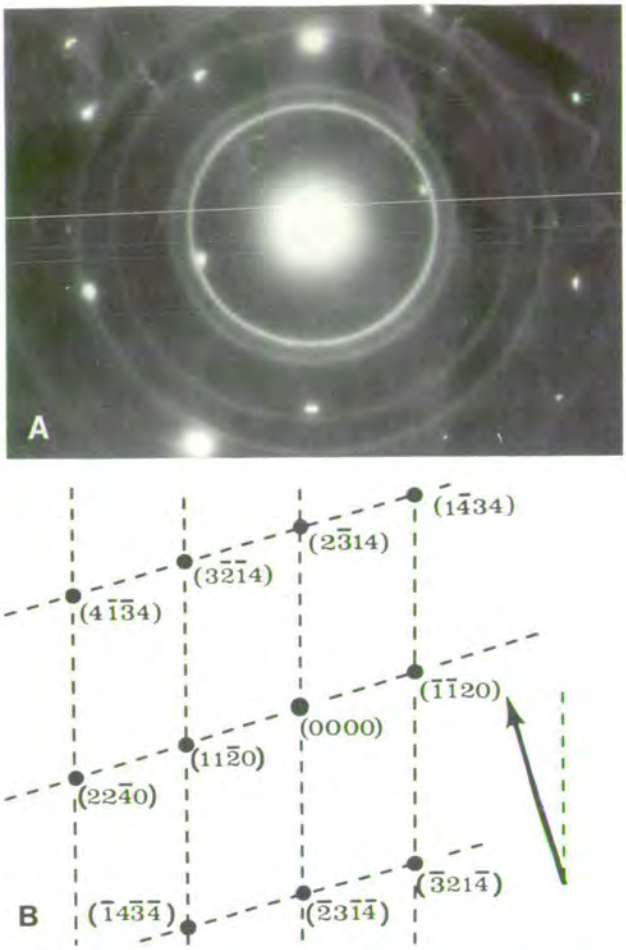

Fig. 2. A Electron diffraction pattern from a thin section prepared from the palisade layer by chemical thinning; single-crystal calcite spots with rings from the gold coating. B Analysis of calcite diffraction pattern illustrated in A; arrow represents the projection of the c-axis in the plane of the diagram

(AB in the diagram) which represents directions ~ 60° to the (0001) pole. The observation that there is an equal division of surface normals inside the outside curve AB confirms the general lack of any preferred orientation.

In the stereographic projection illustrated in Fig. 4 are plotted the electron diffraction results obtained from studies on thinned sections of the surface layer of the eggshell; 19 areas were analyzed using 8 specimens. The data show a tendency for the {10$\bar{1}$4} poles to lie perpendicular to the specimen surface, i.e., for {10$\bar{1}$4} planes to lie parallel to the surface.

Ion-Beam Thinned Samples

Samples of eggshell thinned by the ion-beam technique gave much more satisfactory specimens. Figure 5A, an optical micrograph viewed in direct

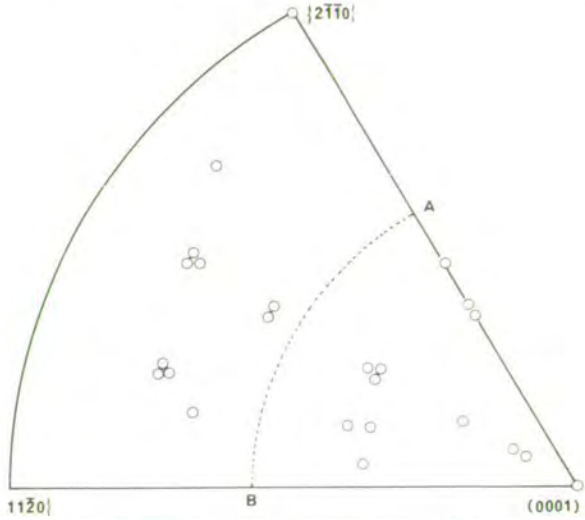

Fig. 3. Orientations in the palisade layer, (0001) stereographic projection; broken line *AB* divides the reference sphere into two equal areas

light, shows an area adjoining the edge of the perforated specimen approximating in size that of a single column of the palisade layer. Figure 5B shows the same area after rotation between crossed polars, differences in contrast clearly indicating a system of boundaries separating crystallites of 20 to 30 μm in diameter. A small part of this area is illustrated in an electron micrograph, Fig. 6, and a number of holes (vesicles) are evident approximately several hundred nanometers in diameter. Based on the information contained within polarized light micrographs (see, for example, Fig. 5B), 14 crystallites in this area of specimen were analyzed using

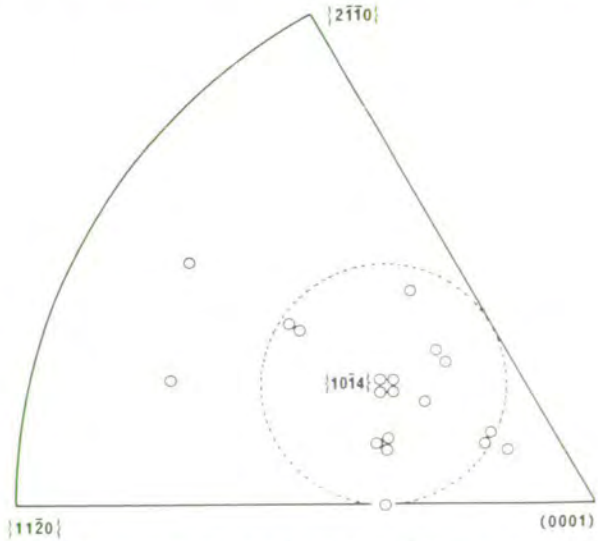

Fig. 4. Orientations in the surface layer; broken circle represents 20° spread about the {10$\bar{1}$4} pole

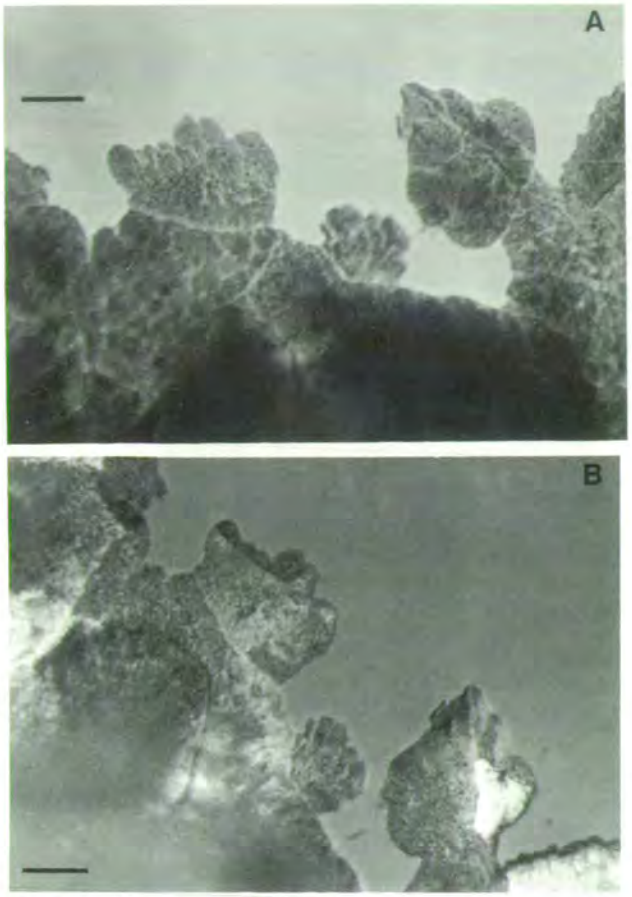

Fig. 5. **A** Section from palisade layer prepared by ion-beam thinning, bar marker represents 20 μm, optical micrograph. **B** Same as **A** but rotated, polarized light

selected area diffraction. The results are given in the stereogram (Fig. 7) and show a small degree of preferred orientation. Most of the points may be included within the broken lines, indicating that the c-axes of individual crystallites lie within the range 30° ± 18° to the surface perpendicular. We show in Fig. 8 the diffraction analyses as related to specific areas of the specimen; the broken lines indicate crystallite boundaries as deduced from polarized light analyses. The arrows indicate the c-axes projected onto the plane of the diagram while the tilts of the respective c-axes are given in the legend.

Discussion

We first refer to our observations on the microstructure of an individual calcite column of the palisade layer and then discuss our results on a number of different columns together with the implications concerning crystal growth.

Each column of calcite consisted of crystallites of

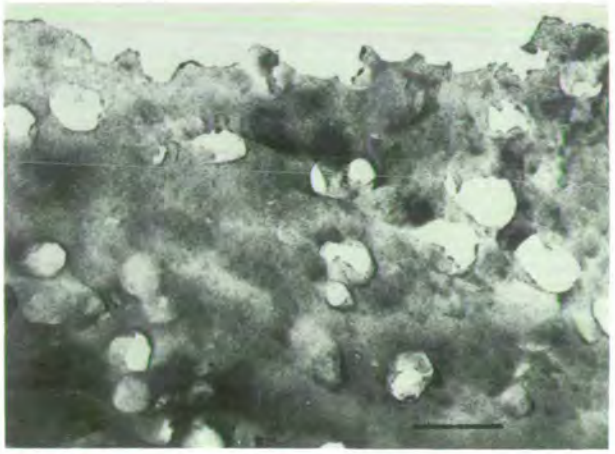

Fig. 6. Same as Fig. 5A but transmission electron micrograph: bar marker represents 1 μm

diameter 20 to 30 μm, slightly larger than the values of 10 to 15 μm reported by Terepka [3]. Some alignment of crystallites comprising a single column was found, their orientations lying between ± 18° of the average crystal direction for the column. With the column examined in detail (see Figs. 7 and 8), the c-axes of the crystallites were inclined at angles between 12° and 48° to the surface normal of the eggshell. When, however, results from a number of different columns were collated, there was no evidence suggesting any preferred orientation in the palisade layer as a whole. These results are in disagreement with previously published work. Certainly the conclusion of both Terepka [3] and Schmidt [4], based on polarized light studies, that

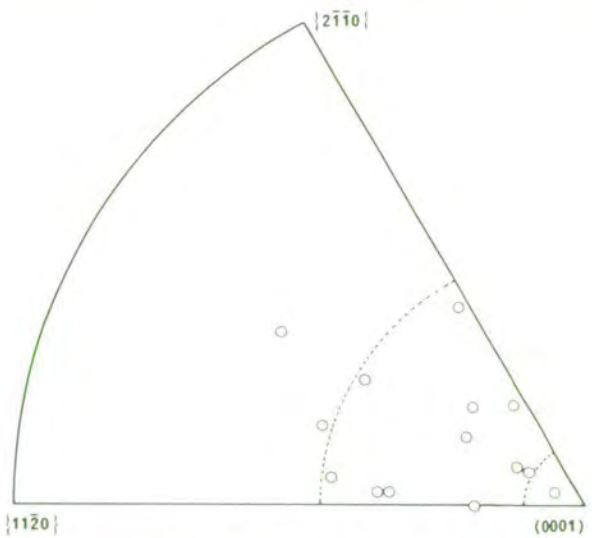

Fig. 7. Orientations in the palisade layer, mostly from a single column, (0001) stereographic projection; broken lines represent angles at 12° and at 48° to the c-axis

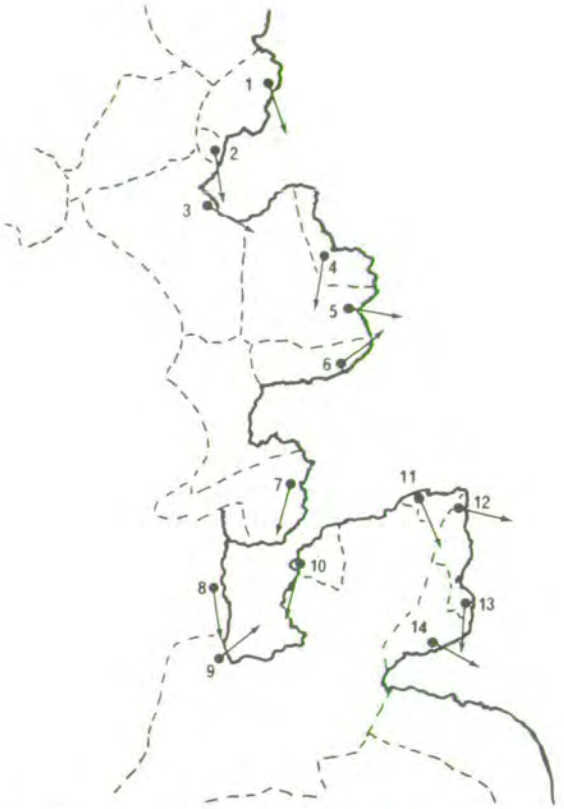

Fig. 8. Diagram of area illustrated in Fig. 5 showing crystallite boundaries *(broken lines)*; numbers indicate areas analyzed and arrows represent the projection of the c-axis, in the plane of the diagram. The angles between the respective c-axes and the surface normal are as follows: *1*, 25°; *2*, 45°; *3*, 38°; *4*, 26°; *5*, 45°; *6*, 52°; *7*, 30°; *8*, 14°; *9*, 16°; *10*, 30°; *11*, 23°; *12*, 39°; *13*, 27°; *14*, 48°

the column axes are parallel to the c-axis of the calcite lattice appears to be incorrect. In fact, Terepka's parallel X-ray studies imply little preferred orientation, in contradiction to his interpretation of polarized light data. The more detailed X-ray diffraction study carried out by Cain and Heyn showed that the calcite crystals comprising the cone layer were randomly oriented but that the large crystal columns of the palisade layer exhibited preferred orientation such that the c-axes of different crystals were aligned within the range 12° to 44° to the surface normal. These results are similar to our electron diffraction data obtained from an essentially single column of the palisade layer, see Fig. 7, where the inclination of c-axes of crystallites was found to range from 12° to 48°. However, the authors do not mention the size of beam used in the X-ray transmission studies, i.e., the number of crystal columns included in any one X-ray diffractogram, and although the X-ray patterns are consistent with diffraction from numerous crystals, this may well be attributable to the large number of crystallites contributing to a single column. Indeed, their estimate from X-ray data of a crystal size of $\sim 200\ \mu m$ is questionable and they appear to have been unaware that each column contains small, differently oriented crystallites.

The only region where we detected any preferred orientation was the surface crystal layer, and here the evidence indicated that $\{10\bar{1}4\}$ planes tended to lie parallel to the eggshell surface. These results are in close agreement with the X-ray diffraction studies of Favejee et al. [7] on the surface layer (of the hen's eggshell); these investigators also noted a tendency for $\{10\bar{1}4\}$ planes to lie parallel to the surface. The degree of preferred orientation was, however, not high. Favejee et al. reported that the c-axes formed a cone of semi-apical angle $\simeq 45°$ with a spread of $\pm 20°$. Figure 4, our electron diffraction data plotted on a pole figure, includes a broken circle to represent a 20° spread about the $\{10\bar{1}4\}$ pole. Most results are seen to lie within this circle, in accord with Favejee's X-ray diffraction findings.

Apart from the present work, the only other electron diffraction studies on eggshells appear to be those of Veress et al. [9] and Quintana and Sandoz [10], but neither of these studies involves the eggshell of the domestic fowl. Veress et al. gave some data on the goose eggshell from an examination in transmission of thin sections prepared by ultramicrotomy but merely confirmed the presence of the calcite form of calcium carbonate. Quintana and Sandoz, in studies of ultramicrotomed sections from the shell of the Japanese quail, identified some of the crystallites in the palisade layer as having the c-axis inclined at 45° and at 60° to the shell surface and stated that the orientation axis persisted through the cone layer. However, their diffraction analyses appear to have been confined to a series of crystallites within a single column and are insufficient to draw any conclusion concerning the existence of a preferred orientation in the palisade layer as a whole.

Let us, therefore, consider the earlier stages of calcite growth in order to explain the present findings concerning the palisade layer. Now Quintana and Sandoz showed that the cone layer included small crystals in random orientation as did Cain and Heyn in their studies of the hen's egg. The randomness suggests the nucleation sites for calcite growth have little orientational relationship one with another. This perhaps is expected from the organic nature of the fibrous membrane on which the calcite grows. The nuclei are well separated and their number, which is probably genetically regulated [11], controls the number of columns which develop later in the deposition process. Although no detailed information is available on the fine structure of the

crystal nucleus, Creger et al. [12] showed micrographs suggesting that it may consist of a group of microcrystals. During shell development the microcrystals then grow outward in all directions, some growing in preference to others. There is a tendency for the external calcite surfaces to consist of low-energy faces, but, due to the great variety of forms and habits exhibited by calcite crystals [13], it is likely that no strong preferred orientation emerges as the crystals develop into the cone layer. Once the cones have impinged on one another to form a continuous calcite layer, further growth is limited to those crystals still in contact with the liquid. The lower regions of the crystal adjacent to the shell membranes cease to develop as the supply of liquid becomes exhausted, whereas the outer regions of the shell continue to grow although the number of crystals participating is reduced.

Now the palisade layer starts, and since the calcite crystals were initially oriented randomly, we expect that the columnar crystals which develop from them show a similar lack of orientation relationship to one another. The small degree of preferred orientation observed for crystallites composing an individual column may be accounted for by assuming that the microcrystals within a particular nucleation center are influenced by their neighbors in the group such that low-angle boundaries rather than high-angle boundaries separate them.

The fact that the surface layer is much more finely crystalline than the palisade layer indicates that calcite deposition conditions have changed. Changes in the structure of the outer shell layer of different avian species have been referred to on a number of occasions. For example, vaterite, a different polymorph of calcium carbonate, was identified in certain sea birds [14] while amorphous calcium phosphate was detected in the surface regions of the Great Crested Grebe [15]. In the hen's egg the crystal structure remains the same although the crystal size is reduced, and some preferred orientation is developed such that $\{10\bar{1}4\}$ planes of the calcite lattice tend to be aligned parallel to the shell surface. Presumably the $\{10\bar{1}4\}$ planes present a surface to the liquid which is of low energy and therefore favored. Certainly these are the planes of easy cleavage in the calcite structure. Indeed, it could be argued that if this crystal orientation were common to the whole palisade layer, the shell would exhibit an undesirable tendency to cleave in directions parallel to the surface.

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References

1. Simons, P.C.M., Wiertz, G.: Notes on the structure of shell and membranes of the hen's egg; A study with the scanning electron microscope, Ann. Biol. Anim. Biochem. Biophys. **10**:31–49, 1970
2. Simons, P.C.M.: Ultrastructure of the Hen Eggshell and Its Physiological Interpretation. Centre for Agricultural Publishing and Documentation, Wageningen, 1971
3. Terepka, A.R.: Structure and calcification in avian egg shell, Exp. Cell Res. **30**:171–182, 1963
4. Schmidt, W.J.: Über die Struktur einiger abnormer Vogel-Eischalen nebst Bemerkungen zu neueren Auffassungen betreffend Bau und Bildung der Kalkschale, Z. Morphol. Ökol. Tiere. **53**:311–361, 1964
5. Winchell, H.: The unit cells of calcite, Am. J. Sci. **254**:65–70, 1956
6. Cain, C.J., Heyn, A.N.J.: X-Ray diffraction studies of the crystalline structure of the avian egg shell, Biophys. J. **4**:23–39, 1964
7. Favejee, J.Ch.L., Van der Plas, L., Schoorl, R., Floor, P.: X-Ray diffraction of the crystalline structure of the avian egg shell: some critical remarks, Biophys. J. **5**:359–361, 1965
8. Heyn, A.N.J.: The crystalline structure of calcium carbonate in the avian egg shell, J. Ultrastruct. Res. **8**:176–188, 1963
9. Veress, I., Szép, I., Bidlo, G.: Examination of the fine structure of goose egg shells by electron microscopy and x-ray diffraction, Baromfiipar **1**:35–46, 1972
10. Quintana, C., Sandoz, D.: Coquille de l'oeuf de caille: étude ultrastructurale et cristallographique, Calcif. Tissue Res. **25**:145–159, 1978
11. Krueger, W.F., cited in [12]
12. Creger, C.R., Phillips, H., Scott, J.T.: Formation of an egg shell, Poult. Sci. **55**:1717–1723, 1976
13. Dana E.S.: A Textbook of Minerology. John Wiley & Sons, New York, 1932
14. Tullett, S.G., Board, R.G., Love, G., Perrott, H.R., Scott, V.D.: Vaterite deposition during eggshell formation in cormorant, gannet and shag and in "shell-less" eggs of the domestic fowl, Acta Zool. (Stockh.) **57**:79–87, 1976
15. Board, R.G., Perrott, H.R., Love, G., Scott, V.D.: The phosphate-rich cover on the eggshells of the Great Crested Grebe, Calcif. Tissue Int. (submitted)

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Porosity of the Avian Eggshell[1]

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SYNOPSIS. The avian eggshell is considered as a mediating boundary that operates along with the nest microenvironment and the behaviour of the brooding parent(s) to isolate the embryo from the external environment. Particular importance is attached to shell modifications that could assure that the pores are not flooded or blocked with debris. It is proposed that the mechanical properties of the shell that ensure the integrity of the diffusion pathways throughout incubation are of equal importance to those that protect the embryo from mechanical damage. Five broad categories of pore types in avian eggshells have been defined: 1) simple pore systems—a tube open at both ends traverses the true shell; 2) occluded pore systems—the outer surface of the shell is coated with featureless material of unknown origin and chemical composition; fissures in this material presumably permit gaseous diffusion; 3) plugged pore systems—the outer orifice of the pore orifice contains a plug of organic or crystalline inorganic material; 4) capped pore systems—the outer surface of the true shell and pore orifices are covered with a stratum of spheres formed from organic material, vaterite or non-crystalline materials rich in calcium and phosporus; 5) reticulate pores—the outer portion of the palisade layer is modified so that a plexus of tubules comprises much of the shell. Although five pore systems have been defined so far, all probably share a common origin in that cones formed in the distal part of the isthmus provide not only the opportunity for pore formation but also foundation for the palisade layer.

INTRODUCTION

Since the pioneering work of Von Nathusius (1821–1899, see Tyler, 1964*a*) avian eggshells have been the subject of many studies dealing with structure rather than function. Recent investigations with the electron microscope have revealed a diverse range of pore types and (*e.g.*, Becking, 1975; Board *et al.*, 1977) also provided novel information about the distribution of elements in the shell (Board and Love, 1980). An important stimulus for investigations of shell form and function came from the classical work of Rahn and his collaborators (*e.g.*, Ar and Rahn, 1978). Not only were they able to quantify the contribution of the pores to the diffusive flow of gases and water vapour but, from comparative studies of many species of birds and investigations of eggs brooded at some environmental extreme (*e.g.*, low barometric pressure), they drew attention to mechanisms that lead to eggshells being endowed with a porosity commensurate with environmental demands.

In this article we discuss the details of fine structure in the context of the overall contribution of the shell to the successful development of the embryo (Fig. 1). Questions are raised concerning the mechanisms operating in the shell gland at the time of crystal growth and in the distal part of the isthmus of the oviduct at the time of cone formation.

THE ROLE OF THE EGGSHELL

We are of the opinion that the avian eggshell is a mediating boundary which, together with other features, contributes to the isolation of the embryo from the nest/bulk environment (Fig. 1). The many modifications of shell structure discussed in this chapter have probably evolved so that the pore canals are not flooded, blocked with debris or infected with microorganisms

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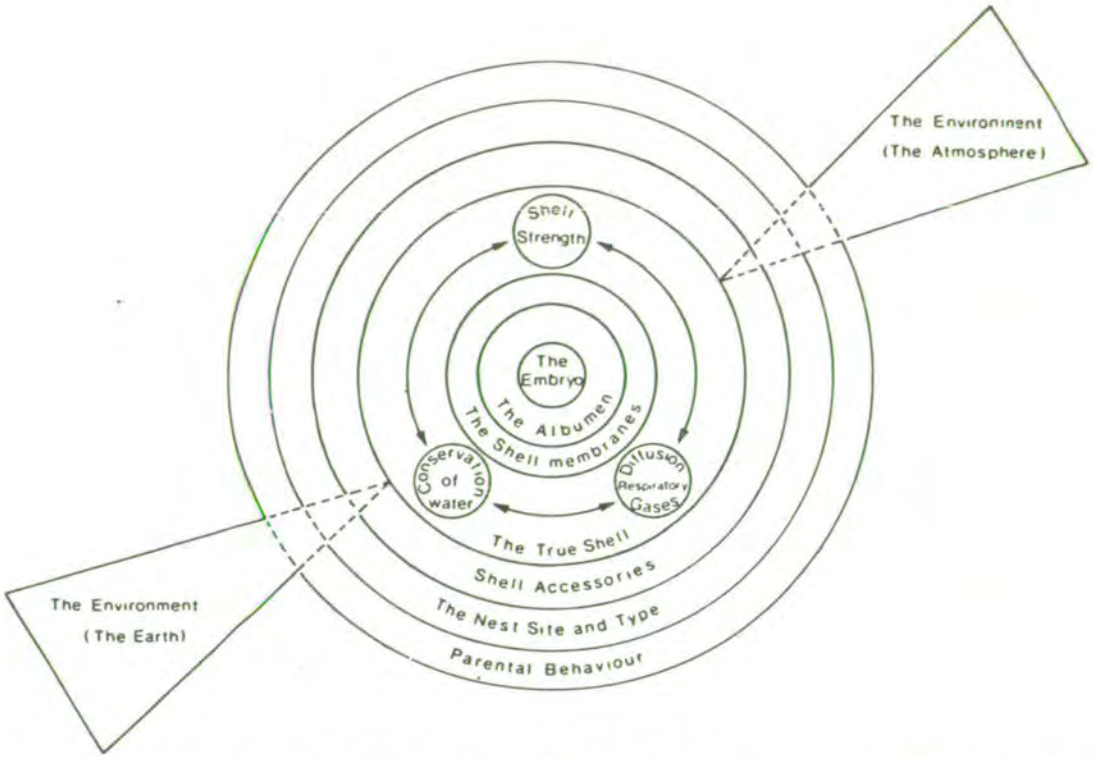

FIG. 1. A schematic representation of the roles of the shell and shell accessory materials in isolating the embryo from the environment.

(Board, 1980). In some cases also the modifications may provide protection against mechanical damage that would destroy the diffusion network in the shell.

SHELL STRUCTURE AND COMPOSITION

The shell of the avian egg is composed mainly of inorganic materials (*ca.* 95%) with an organic matrix (*ca.* 4%) and water (Romanoff and Romanoff, 1949; Erben, 1970; Simons, 1971).

Nomenclature

A diverse nomenclature has evolved to label the various components of the shell (Schmidt, 1962; Tyler, 1969*a*; Simons, 1971). Of the names given in Figure 2, we will use cone layer for the innermost part and palisade layer for the bulk of the shell and follow Tyler (1969*a*) by considering these two to be the components of the true shell. The tips of the cones (the basal caps) make intimate contact with the surface of the outer shell membrane that envelops the yolk and albumen. The outer part of the palisade layer is commonly formed from dense crystalline material, an arrangement that is particularly noticeable in the electron microscope (Becking, 1975; Quintana and Sandoz, 1978). The outer surface of the true shell may be covered with a distinct layer of either inorganic or organic material, termed cover and cuticle in the older literature, which we term "shell accessory materials." A pore canal originates between the cones (Tullett, 1975), extends radially across the palisade layer and terminates at the outer surface of the true shell. With some shells, modifications of the outer part of the palisade layer lead to the pore canal "losing its identity" before reaching the outer surface of the shell (Fig. 2).

Calcium carbonate

There is no dispute that, apart from a trace of aragonite (Erben, 1970), calcite is the main component of the true shell. As

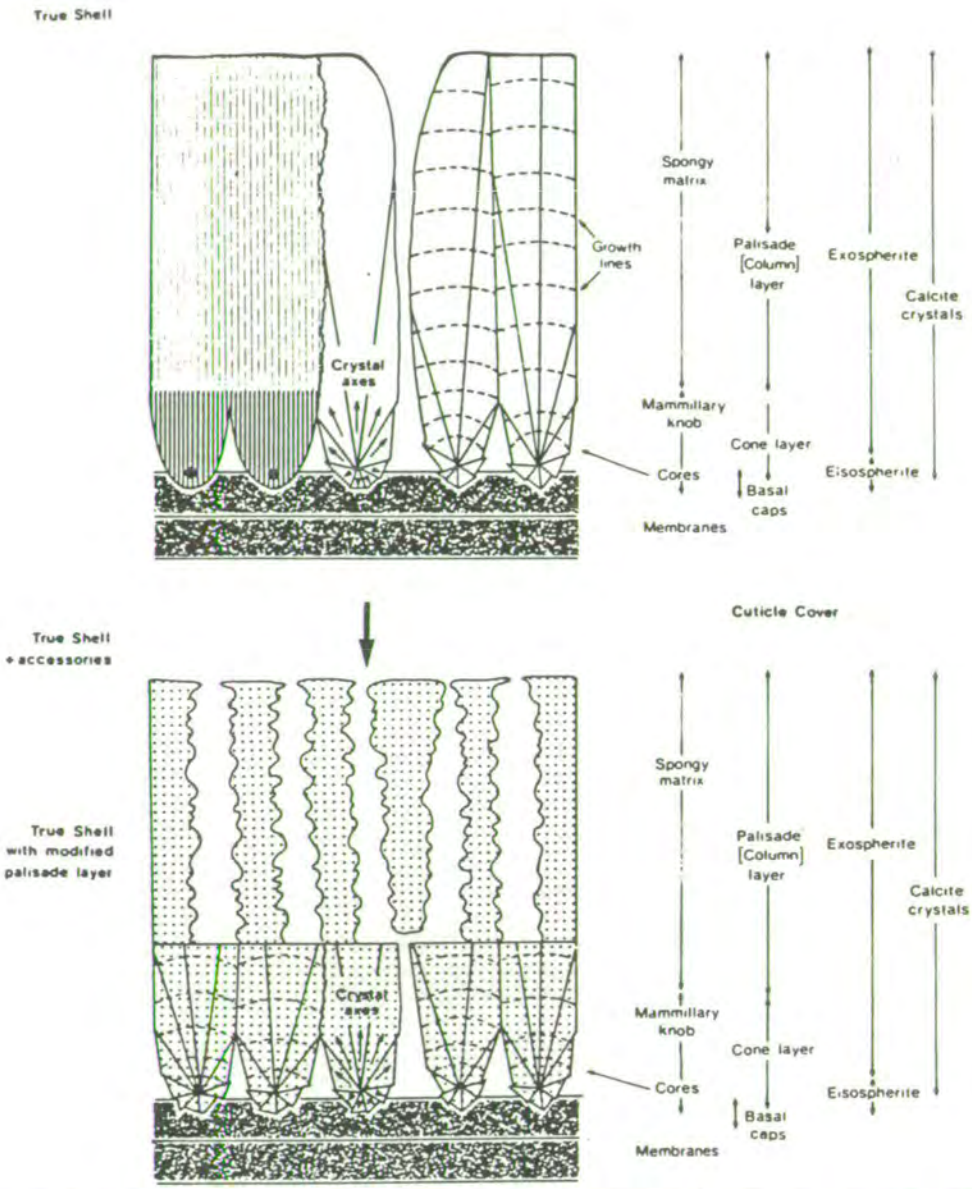

FIG. 2. Stylized drawing of radial section of eggshell showing transition from true shell → true shell with accessory materials → true shell with modified palisade layer. Terminology taken from Schmidt (1962), Tyler (1969*a*), and Simons (1971).

yet, however, no consensus exists on the crystallographic details of the arrangement of calcite, especially in the palisade layer (Tyler, 1969*a*). When thin radial sections of shell are viewed with polarized light, the palisade layer appears to be composed of long radial columns of crystal. That they were extinguished in turn as the specimen was rotated was taken as evidence for their being single crystal with the c-axis of the calcite unit cell (hexagonal) perpendicular to the shell surface (Terepka, 1963). This was contradicted by Masshoff and Stolpmann (1961) who concluded from studies of the shell of domestic fowls (*Gallus domesticus*) that each column

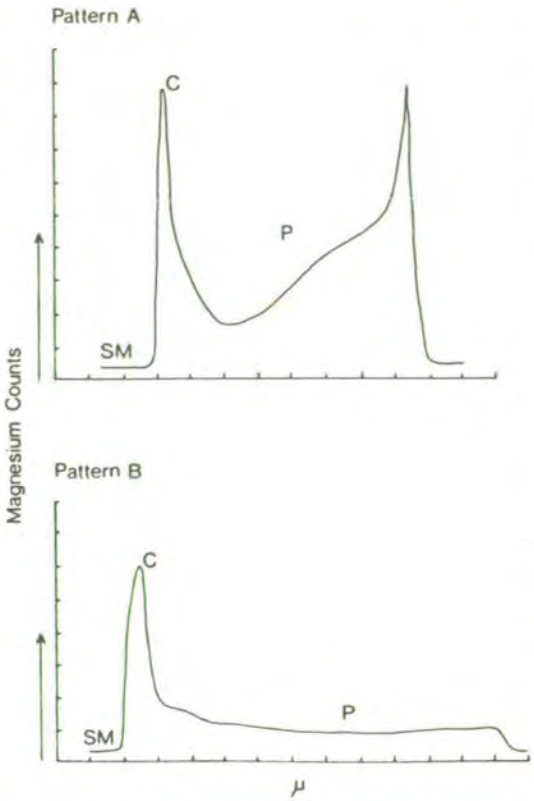

FIG. 3. Patterns of magnesium distribution in radial sections of eggshells as determined by electron probe microanalysis (Board and Love, 1980). For further details, see text. SM, shell membrane; C, cone; P, palisade.

consists of minute crystals embedded in an organic matrix. Subsequent studies of the orientation of calcite have given different results also. For example, the c-axis is reported to make an angle of *ca.* 20–50° with the normal to the shell surface (Heyn, 1963) or 28 ± 16° (Cain and Heyn, 1964). However, Favejee *et al.* (1965) found a random arrangement with a tendency for the c-axis to be inclined at 45° to the shell surface, although these results were obtained only from the outer surface of the shell. Quintana and Sandoz (1978) reported an angle of 45° and 60° in the shell of the Japanese quail, *Coturnix coturnix*, while our own studies on chemically thinned sections of the shell of hen's eggs have shown little preferred orientation apart from some alignment of $\{10\bar{1}4\}$ planes parallel to the surface in the outer layers. However, we are of the opinion that little useful purpose would be served by further detailed crystallographic analysis unless it is done in conjunction with studies of shell formation in which emphasis is given to factors governing the initiation of crystal deposition, and the role of organic fibres as nucleation sites.

Magnesium and phosphate

Magnesium and phosphate are minor constituents of avian eggshells, *i.e.*, 0.84–1.88% $MgCO_3$, 0.55–0.75% "tricalcium phosphate" as compared to 97.37–98.84% $CaCO_3$/shell (Romanoff and Romanoff, 1949). Even in these quantities, however, they could modify crystal growth and impart specific properties to the shell.

In the eggshells of the domestic fowl (Brooks and Hale, 1955; Itoh and Hatano, 1964) and Japanese quail, *Coturnix coturnix* (Sandoz and Quintana, 1978), the magnesium concentration increases progressively from the lowest level at the innermost part of the palisade layer to a maximum at the outer edge. In a survey of eggshells by electron probe microanalysis (Board and Love, 1980), Pattern A (Fig. 3) was common to all eggshells of members of the Galliformes, while Pattern B (Fig. 3) was characteristic of 19 other orders of birds. The lily-trotter, *Micropara capensis*, however, exhibited Pattern A, whereas other members of its order, Charidriiformes (Sibley and Ahlquist, 1972), gave Pattern B.

A relationship was found between the magnesium content and the strength and hardness of the eggshell of the domestic hen (Brooks and Hale, 1955). Shells of average strength had a magnesium level of 0.404% whereas strong and weak shells had 0.440% and 0.386%, respectively. It has been noted also (Longcore *et al.*, 1971) that DDE in a bird's diet, which can lead to increased concentrations of magnesium, altered the mechanical properties of the eggshell. Fox (1976) found that the "dented" eggshells of common terns, *Sterna hirundo*, contained 2.10% whereas the shells from which chicks hatched had 1.54%

magnesium; he also noted that eggs laid before the use of DDE in agriculture had 1.80% magnesium. When discussing this evidence, Cooke (1979) expressed the view, based on the work of Kitano *et al.* (1976), that magnesium may be an important cause of DDE-related eggshell thinning by poisoning calcite formation. This element is known to retard calcite crystal growth and cause the formation of magnesian calcite, a more soluble material than pure calcite (Berner, 1975). The latter observation is of particular interest with respect to the reclamation of calcium from the shell since it has been postulated (Quintana and Sandoz, 1978) that magnesium in the cone layer of the eggshells of Japanese quail, *C. coturnix*, may contribute to this process.

The distribution of phosphate in the hen's eggshell parallels that of magnesium (Itoh and Hatano, 1964). The concentration of phosphate tends to be higher in eggshells formed by birds receiving a DDT-contaminated diet. Fox (1976) found 0.63% phosphorus in "dented" eggshells and 0.86% in those containing dead embryos of the common tern, *Sterna hirundo*, after exposure to DDT. Eggshells collected before the introduction (pre-1945) of the pesticide contained only 0.27% phosphorus, a level similar to the 0.24% found in eggshells from which young had hatched. As phosphate is a potent poison of calcite formation (Simkiss, 1964), it has been proposed (Cooke, 1979) that it may also be involved in DDT-related eggshell thinning. Indeed, Bachra *et al.* (1963) noted that calcium carbonate precipitation was prevented by the presence of phosphate ions. When attempting to explain the abrupt change from calcite to vaterite, a shell accessory material, on the eggshell of the gannet, *Sula bassana*, Tullett *et al.* (1976) suggested that phosphate may poison calcite but not vaterite formation. Furthermore, we have noted (Board *et al.*, unpublished observations) high levels of phosphorus in the non-crystalline calcium-rich shell accessory materials on the eggs of the great-crested grebe, *Podiceps cristatus*, and the Chilean flamingo, *Phoenicopterus chilensis*.

Organic matrix

Fibres are evident in decalcified shells (Masshoff and Stolpmann, 1961; Simons and Weirtz, 1963; Wyburn *et al.*, 1973). Although minute amounts of organic material have been seen on partially formed shells taken *post mortem* from the oviduct (Fujii, 1974) or on prematurely laid eggs (Simons, 1971), Wyburn *et al.* (1973) state that they never found an egg in the shell gland with an uncalcified matrix. Recent studies (*e.g.*, Hamm *et al.*, 1977) are yielding information on the process of crystallization; they have not yet shed light on the role of the organic matrix in determining shell formation. Moreover, our observations (Bond *et al.*, 1980) that many eggshells are tough with a degree of flexibility implies that the matrix may function not only as a framework upon which crystals grow but also as an important component of the mechanical properties of the shell.

THE PORES

Observations made with the light microscope and plastic models indicate that, apart from a few eggshells having lanceolate or forked pores, the basic morphology of a pore canal resembles that of a straight post-horn (Tyler, 1964*b*, 1965, 1966, 1969*b*; Tyler and Simkiss, 1959). The exceptions have been noted mainly in thick shells such as those of the swans and ratites. The pores in the eggshell of ostriches, *Struthio camellus*, are extensively branched and canals sharing a common origin in the cone layer terminate in a saucer-shaped depression in the surface of the shell (Tyler and Simkiss, 1959). Those in the eggshells of rheas are much less branched; the few branches sharing a common origin terminate in a groove in the outer layer of the shell (Tyler and Fowler, 1979). The mechanisms responsible for branching are not known but shell thickness appears to be an important factor.

Discussions of the contribution of the shell to the successful growth of the embryo and to the importance of diffusion pathways suggest that the pore canal ought to be considered in relation to the shell accessory materials and to modifications of

the palisade layer (Fig. 2). Indeed such relationships form the basis of the arbitrary classification given in Figure 4.

The simplest pore system is found in the eggshells of pigeons and doves (Board *et al.*, 1977). Pore canals, open at both ends, traverse the true shell (Fig. 4).

The occluded pore system (Fig. 4) can be considered a derivative of the simple pore form. Accessory materials of unknown form and chemical composition form a featureless skin on the shell's outer surface. Fissures in the material traversing the outer pore orifice are presumably the pathways for gaseous diffusion.

Plugged pore systems have been found in the eggshells of the greater rhea, *Rhea americana* (Board *et al.*, 1977), the lily-trotter, *Micropara capensis*, four species of tinamou (Board and Perrott, 1979*a*) and probably the cuckoo, *Cuculus canorus* (Becking, 1975). The plug occurs in different forms. The organic plug of the eggshell of the lily-trotter is rich in iron and lacks fine structure other than a vague ridging of its surface; the outer surface of the plug is below that of the shell. Spheres and fibres, both rich in sulphur, make up the bulk of the plug in the tinamou eggshell, the featureless outer surface being flush with the shell surface. We have noted plugs of similar morphology in preliminary studies of the shell of the Kiwi, *Apteryx*. The plugs in another ratite eggshell, the greater rhea, *Rhea americana*, are apparently formed from irregularly shaped crystalline material; cracks between the crystalline material presumably link the lumen of the canals to the outside of the egg. The plug of the eggshell of the lily-trotter appears to be a loose-fitting cone in a campanulate orifice and it is assumed that the space between the two provides a diffusion pathway. The plugs in the tinamou eggshell appear to be formed concomitantly with the outer crystalline layer; many of the spheres at the periphery of the plug are partially lodged in cavities in the outer band of crystalline material. The cracks in the featureless surface of these plugs connect the outside of the shell, the spaces between the spheres and fibres of the plug, and the lumen of the canal. Lack (1968) cited the lily-trotter's eggshell as an example of one that had probably been adapted to prevent water logging. Board and Perrott (1979*a*) speculated that (i) the cone-shaped plug may operate as a valve should a positive hydrostatic pressure be applied to the shell and (ii) the honeycombed wall of the pore could not provide a contact angle suitable for the capillary movement of water.

The outer surface of the shells with capped pore systems (Fig. 4) is covered with "accessory materials" in the form of a stratum of spheres. The stratum can either bridge the outer pore orifice or extend down into the pore canals (Board *et al.*, 1977). The spheres are mainly organic, such as those on the shells of the eggs of domestic fowl (*e.g.*, Cooke and Balch, 1970), or mainly inorganic. In some species the accessory material is crystalline principally vaterite, *e.g.*, members of the Pelecaniformes and *Guira guira* and *Crotophagi ani* (Board and Perrott, 1979*b*). Non-crystalline spheres, rich in calcium and phosphorus, are found on the great-crested grebe, *Podiceps cristatus*, and the Chilean flamingo, *P. chilensis* (Board *et al.*, unpublished observations). A tortuous diffusion pathway must be a feature of capped pores. Radial fissures in the stratum of the spheres covering the pore orifice in eggs of the domestic fowl are presumably the major pathway for diffusion. We have noted that the spheres on the outer surface of the eggs of the Guinea fowl, *Numidia meleagris*, become coated with debris when brooded by domestic hens. Likewise the shells of the eggs of wild birds are frequently soiled. Could it be that through soiling, the lumen of the pore canal exchanges gases with the void spaces of the stratum of spheres and that these spaces

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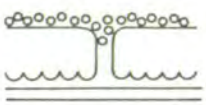
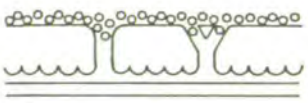
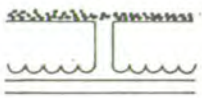
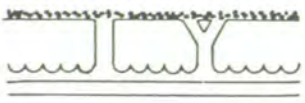
FIG. 4. Pore systems in avian eggshells arranged in order of increasing complexity. This figure, first published by Board (1980), is based on the observations of Board and Perrott (1979*a*, *b*) and Board *et al.* (1977). Reproduced by kind permission of the Editors of the *Journal of Applied Bacteriology*.

PORES

Outer orifice	Canal	
Open	Unbranched	
	Unbranched and branched	
Occluded	Unbranched	
	Unbranched and branched	
Plugged	Unbranched	
	Unbranched and branched	
Capped	Unbranched	
	Unbranched and branched	
Reticulate	Unbranched	
	Unbranched and branched	

then exchange with the nest environment? It has been suggested elsewhere (Board *et al.*, 1977) that turning the eggs in the nest may play an important role in cleaning the shell surface and thus maintaining diffusion pathways.

The reticulate pore system (Fig. 4) also creates a tortuous pathway for gaseous diffusion. The pore canals retain their identity for only a part of the total width of the shell because the outer part of the palisade layer is extensively modified. This modification leads to a network of channels surrounding the entire shell with small holes in the shell surface venting the channels to the exterior. Such pore systems have been found in the egg shells of cassowary, *Casuarius casuarius,* emu, *Dromaius nouvaehollandiae,* storks (open-billed, *Anastomus oscitans*; white, *Ciconia alba*; yellow-billed, *Mycteria ibis*) and members of the Accipitridae except Lammergeier and Egyptian vulture.

In summary it is considered that, apart from the eggshells of ratites, studies of shell structure have not provided sufficient information to support a discussion on the phylogenetic implications of these different pore types. Although the ratites are considered by some to be monophyletic (Cracraft, 1973; Prager *et al.*, 1976), their eggshells exhibit a diversity of form. Thus eggshells of the ostrich, *Struthio camellus,* contain multiple-branched pores with open orifices clustered in saucer-shaped depressions in the shell surface (Tyler and Simkiss, 1959). The pores in the rhea eggshell branch less profusely and the orifices are contained in grooves in the surface (Tyler and Fowler, 1979). The orifices of the greater rhea, *Rhea americana,* are plugged with crystalline material whereas those of the emu, *D. nouvaehollandiae,* and cassowary, *C. casuarius,* have reticulate pore systems. The tinamou eggshell contains pores plugged with organic material; the Kiwi pore is plugged also but the plugging material has not been characterized.

Pore Formation

The above survey of shell structure and chemical composition provides clues as to the possible causes of some of the pore systems discussed in the preceding section. For example, phosphate was implicated in the switch from calcite in the true shell to vaterite in the "accessory material" on the eggs of the gannet, *Sula bassana* (Tullett *et al.*, 1976), while the interaction of phosphate, bicarbonate and calcium in crystalization (Bachra *et al.*, 1963) indicates that phosphate is involved in the formation of "accessory materials" on the shells of the great-crested grebe, *P. cristatus,* and Chilean flamingo, *Phoenicopterus chilensis.* These observations suggest that the shell gland receives relatively large amounts of phosphate towards the end of shell formation, perhaps as a consequence of the breakdown of skeletal bone. It has been shown that such bone can be mobilised during the night by in-lay domestic hens (Mongin and Sauveur, 1979). As all the pore types shown in Figure 4 are created by conditions in the shell gland at or towards the end of shell formation and as none is identifiable in allometric relationships (Ar and Rahn, 1978), it would seem reasonable to assume that their biological significance is to be found in the general context of the avian eggshell acting as a mediating boundary.

To gain an understanding of the determinants of porosity—and perhaps certain aspects of shell strength (Carter, 1979)—attention has to be given to the formation of the cones and the inner part of the palisade layer. We have observed a well-formed palisade layer immediately above the cone layer in all eggs examined and it would seem that the model of the shell gland which Mongin and Carter (1977) based on their studies of the domestic hen may be generally applicable. Likewise the studies of the relationship between mammillary cores, cones, shell thickness, etc. (Tyler and Fowler, 1978), may provide an explanation for pore initiation not only in "normal" eggshells but also in those adapted for incubation at environmental extremes (Lomholt, 1976; Packard *et al.*, 1977; Rahn *et al.*, 1977; Calder, 1978; Seymour and Rahn, 1978) and in those having a reduced porosity due to DDT (Fox, 1976; Cooke, 1979).

Wyburn *et al.* (1973) demonstrated in histochemical studies that cone formation is initiated in the "red region," the distal 4 cm, of the isthmus of the oviduct. Immediately following the arrival of the egg in this region, fibrous conical projections, the mammillae, are distributed on the external surface of the outer shell membrane, the actual spacing being determined initially by the cohesion of secretions from groups of cells and subsequently by water absorption "plumping" the egg. They directed attention also to dense, round bodies related to the mammillae, identifying them with the mammillary core (Fig. 2), and noted that the first nucleation of calcite crystals occurred on transformed fibres within or in contact with mammillae. From their studies of the eggshells of wild birds, Tyler and Fowler (1978) found an almost perfect linear relationship between core and cone numbers, core and cone junction numbers, and cone and cone junction numbers. They considered, however, that there was no significant relationship between pore numbers and these three parameters, preferring to link pore numbers with shell thickness. In contrast, Tullett (1975) presented evidence that linked pore numbers with the number of mammillae.

There have been many studies of the surface of eggs taken from the oviduct at various stages of calcification (Fujii and Tamura, 1970; Simons, 1971; Fujii, 1974; Creger *et al.*, 1976; Stemberg *et al.*, 1977). Fujii (1974) concluded that "sand-like" granules on the outer surface of the shell membranes were the first stages of shell formation. These grew outwards and eventually fused leaving small spaces some of which remained open to form "air pores." As the cones in the early stages of formation are hexagonal in outline (Fujii and Tamara, 1970), Tullett (1975) argued that space would be filled if their seeding sites were uniformly distributed. To achieve pores, therefore, there is a *prima facie* case for an irregular distribution of seeding sites and, according to Tyler (1956), continuous inward flow of "plumping" fluid. In a comparative study of the eggshell of 59 species of birds, Tullett and Board (1977) found reasonable agreement between total pore area, as determined by the cross sectional area at the narrowest part (where the cones fuse), the number of pores per shell, and the functional pore area calculated from measurement of shell conductance (Ar *et al.*, 1974). They showed also a linear relationship with pores per egg *versus* egg weight, individual pore area *versus* egg weight and shell thickness *versus* cones per unit area, observations that support the views of Tyler and Fowler (1978) that the initial zones of crystallization (the cores) play a role in determining shell thickness, pore numbers and size. Tullett (1978) has suggested that the rate of flow of "plumping fluid" at the time of cone formation could well be an important factor not only in determining the number (and size) of pores per unit area but also in adapting egg porosity to the nest environment. Although the evidence supports the view that cones play an important determinative role in shell form and that the "plumping fluid" may operate as a fine control of pore number and size, further work is needed in this area. It may be concluded, therefore, that attention ought to be focused on the factors influencing the number and distribution of the mammillary cores.

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REFERENCES

Ar, A., C. V. Paganelli, R. B. Reeves, D. G. Greene, and H. Rahn. 1974. The avian egg: Water vapour conductance, shell thickness and functional pore area. Condor 76:153–158.

Ar, A. and H. Rahn. 1978. Interdependence of gas conductance, incubation length and weight of the avian egg. *In* J. Piiper (ed.), *Respiratory function in birds, adult and embryonic*, pp. 227–238. Springer-Verlag, Berlin.

Bachra, B. N., O. R. Trautz, and S. L. Simon. 1963. Precipitation of calcium carbonates and phosphates. I. Spontaneous precipitation of calcium carbonates and phosphates under physiological conditions. Arch. Biochem. Biophys. 103:124–138.

Becking, J. H. 1975. Ultrastructure of avian eggshell. Ibis 117:143–151.

Berner, R. A. 1975. The role of magnesium in the crystal growth of calcite and aragonite from sea water. Geochim. Cosmochim. Acta 39:489–504.

Board, R. G. 1980. The avian eggshell: A resistance network. J. Appl. Bact. 48:303–313.

Board, R. G. and G. Love. 1980. Magnesium distribution in avian eggshells. Comp. Biochem. Physiol. (In press)

Board, R. G. and H. R. Perrott. 1979*a*. Plugged pores of the tinamou (Tinamidae) and jaçana (Jaçanidae) eggshells. Ibis 121:469–474.

Board, R. G. and H. R. Perrott. 1979*b*. Vaterite, a constituent of the eggshells of the non-parasitic cuckoos, *Guira guira* and *Crotophagi ani*. Calc. Tiss. Intnl. 29:63–69.

Board, R. G., S. G. Tullett, and H. R. Perrott. 1977. An arbitrary classification of the pore system of avian eggshells. J. Zool., London 182:251–265.

Bond, G. M., V. D. Scott, R. G. Cooke, and R. G. Board. 1980. Correlation of hatching techniques with mechanical properties of their shells. *In* J. Curry and J. Vincent (eds.), *The mechanical properties of biological materials*. Cambridge University Press, Cambridge. (In press)

Brooks, J. and H. P. Hale. 1955. Strength of the shell of the hen's egg. Nature (London) 175:848–849.

Cain, C. J. and A. N. J. Heyn. 1964. X-ray diffraction studies on the crystalline structure of the avian egg shell. Biophys. J. 4:23–37.

Calder, W. A. 1978. The kiwi: A case of compensating divergence from allometric predictions. *In* J. Piiper (ed.), *Respiratory functions in birds, adult and embryonic*, pp. 239–242. Springer-Verlag, Berlin.

Carter, T. C. 1979. The hen's egg: Evidence of the mechanism relating shell strength to loading rate. Br. Poult. Sci. 20:175–183.

Cooke, A. S. 1979. Changes in eggshell characteristics of the sparrowhawk (*Accipiter nisus*) and peregrine (*Falco peregrinus*) associated with exposure to environmental pollutants during recent decades. J. Zool., London 187:245–263.

Cooke, A. S. and D. A. Balch. 1970. Studies of membrane, mammillary cores and cuticle of the hen eggshell. Br. Poult. Sci. 11:345–352.

Cracraft, J. 1973. Continental drift, paleoclimatology, and the evolution and biogeography of birds. J. Zool., London 169:455–545.

Creger, C. R., H. Philips, and J. Scott. 1976. Studies on the formation of the egg shell. Poult. Sci. 55:1717–1723.

Erben, H. K. 1970. Ultrastrukturen und Mineralisation rezenter und fossiler Eischalen bei Vögeln und Reptilien. Biomineralisation 1:1–66.

Favejee, J. Ch. L., L. van der Plas, R. Schoorl, and P. Floor. 1965. X-ray diffraction of the crystalline structure of the avian eggshell: Some critical remarks. Biophys. J. 5:359–361.

Fox, G. A. 1976. Eggshell quality: Its ecological and physiological significance in a DDT-contaminated common tern population. Wilson Bull. 88:449–475.

Fujii, S. 1974. Further morphological studies on the formation and structure of hen's eggshell by scanning electron microscopy. J. Fac. Fish. Anim. Husb. Hiroshima Univ. 13:29–56.

Fujii, S. and T. Tamura. 1970. Scanning electron microscopy of shell formation in hen's eggs. J. Fac. Fish. Anim. Husb. Hiroshima Univ. 9:65–81.

Hamm, von M., J. Engels, G. Hausmanns, K. Weise, G. Flajs, and G. Krampitz. 1977. Untersuchungen zur Frage des molekularen Mechanismus der Eischalenbildung 6. Mitteilung: Methodik der Isolierung von Ca-Bindungsmolekülen aus Eischalen, Uterussekret und Uterusmucosa von Huhnern und Wachteln. Arch. Geflügelk. 41:212–221.

Heyn, A. N. J. 1963. The calcification of the avian eggshell. Ann. N. Y. Acad. Sci. 109:246–250.

Itoh, H. and T. Hatano. 1964. Variation of magnesium and phosphorus deposition rates during eggshell formation. Poult. Sci. 43:77–80.

Kitano, Y., N. Kanamori, and S. Yoshioka. 1976. Influence of chemical species on the crystal type of calcium carbonate. *In* M. Watabe and K. M. Wilbur (eds.), *The mechanisms of mineralisation in the invertebrates and plants*, pp. 191–202. University of South Carolina Press, Columbia.

Lack, D. 1968. *Ecological adaptations for breeding in birds*. Chapman and Hall, London.

Lomholt, J. P. 1976. Relationship of weight loss to ambient humidity of birds eggs during incubation. J. Comp. Physiol. 105:189–196.

Longcore, J. R., F. B. Samson, J. F. Kreitzer, and J. W. Span. 1971. Changes in mineral composition of eggshells from black ducks and mallards fed DDT in the diet. Bull. Environ. Contam. Toxicol. 6:345–350.

Masshoff, W. and H. J. Stolpmann. 1961. Light and electron microscopic studies of the shell membrane and calcareous shell of the hen's egg. Z. Zellforsch. 55:818–832.

Mongin, P. and N. W. Carter. 1977. Studies on the avian shell gland during egg formation. Br. Poult. Sci. 18:339–351.

Mongin, P. and B. Sauveur. 1979. Plasma inorganic phosphorus concentration during eggshell formation. Br. Poult. Sci. 20:401–412.

Packard, S. C., P. R. Sotherland, and M. J. Packard. 1977. Adaptive reduction in permeability of avian eggshells to water vapour at high altitudes. Nature (London) 266:255–256.

Prager, E. M., A. C. Wilson, D. T. Osuga, and R. E. Feeney. 1976. Evolution of flightless land birds on southern continents: Transferrin comparison shows monophyletic origin of Ratites. J. Mol. Evol. 8:283–294.

Quintana, C. and D. Sandoz. 1978. Coquille de l' oeuf de caille: étude ultrastructurale et crystallographique. Calc. Tiss. Intnl. 25:145–159.

Rahn, H., C. Carey, K. Balmas, B. Bhatia, and C. Paganelli. 1977. Reduction of pore area of the avian eggshell as an adaptation to altitude. Proc. Natl. Acad. Sci. U.S.A. 74:3095–3098.

Romanoff, A. L. and A. J. Romanoff. 1949. *The avian egg*. John Wiley and Sons, New York.

Schmidt, W. J. 1962. Liegt der Eischalenkalk der

Vogel als Submikroskopische Kristallite vor? Z. Zellforsch. 57:848–880.

Seymour, R. S. and H. Rahn. 1978. Gas conductance in the eggshell of the mound-building brush turkey. *In* J. Piiper (ed.), *Respiratory function in birds, adult and embryonic*, pp. 241–246. Springer-Verlag, Berlin.

Sibley, C. G. and J. E. Ahlquist. 1972. A comparative study of the egg white proteins of non-passerine birds. Bulletin no. 39. Peabody Museum of Natural History, Yale University, Connecticut.

Simkiss, K. 1964. Phosphates as crystal poisons of calcification. Biol. Rev. 39:487–505.

Simons, P. C. M. 1971. Ultrastructure of the hen eggshell and its physiological interpretation. Centre for Agricultural Publishing and Documentation, Wageningen.

Simons, P. C. M. and G. Wiertz. 1963. Notes on the structure of membranes and shell in hen's eggs. An electron microscopical study. Z. Zellforsch. Mikrosk. Anat. 59:55–567.

Stemberg, B. H., W. J. Mueller, and R. M. Leach. 1977. Microscopic study of the initial stages of eggshell calcification. Poult. Sci. 56:537–543.

Terepka, A. R. 1963. Organic-inorganic interrelationships in avian eggshells. Exptl. Cell Res. 30:183–192.

Tullett, S. G. 1975. Regulation of avian eggshell porosity. J. Zool., London 177:339–348.

Tullett, S. G. 1978. Pore size versus pore number in avian eggshells. *In* J. Piiper (ed.), *Respiratory function in birds, adult and embryonic*, pp. 217–226. Springer-Verlag, Berlin.

Tullett, S. G. and R. G. Board. 1977. Determinants of avian eggshell porosity. J. Zool., London 183:203–211.

Tullett, S. G., R. G. Board, G. Love, H. R. Perrott, and V. D. Scott. 1976. Vaterite deposition during eggshell formation in the cormorant, gannet, shag, and in "shell-less" eggs of domestic fowl. Acta Zool. (Stockh.) 57:79–87.

Tyler, C. 1956. Studies on eggshells. VII. Some aspects of structure as shown by plastic models. J. Sci. Food Agr. 7:483–493.

Tyler, C. 1964*a*. Wilhelm von Nathusius, 1821–1899, on *Avian eggshells*. A translated and edited version of his work. The University, Reading.

Tyler, C. 1964*b*. A study of the eggshells of the Anatidae. Proc. Zool. Soc. Lond. 142:547–583.

Tyler, C. 1965. A study of the eggshells of Sphenisciformes. J. Zool., London 147:1–19.

Tyler, C. 1966. A study of the eggshells of the Falconiformes. J. Zool., London 150:413–425.

Tyler, C. 1969*a*. Avian eggshells: Their structure and characteristics. Int. J. Gen. Exp. Zool. 40:82–127.

Tyler, C. 1969*b*. A study of the eggshells of the Gaviiformes, Procellariiformes, Podicipitiformes and Pelecaniformes. J. Zool., London 158:395–412.

Tyler, C. and S. Fowler. 1978. The distribution of organic cores, cones, cone junctions, and pores in the eggshells of wild birds. J. Zool., London 186:1–14.

Tyler, C. and S. Fowler. 1979. The size, shape and orientation of pore grooves in eggshells of *Rhea* sp. J. Zool., London 187:283–290.

Tyler, C. and K. Simkiss. 1959. A study of the eggshells of ratite birds. Proc. Zool. Soc. London 133:201–243.

Wyburn, G. M., H. S. Johnston, M. H. Draper, and M. F. Davidson. 1973. The ultrastructure of the shell forming region of the oviduct and the development of the shell of *Gallus domesticus*. Quart. J. Exp. Physiol. 58:143–151.

SECTION 3

PROPERTIES AND FUNCTIONS OF THE SHELL AND SHELL MEMBRANES

It was noted in the Introduction that the avian eggshell and its two lining membranes contribute in many ways to the wellbeing of the developing embryo. In practice, there has been a tendency for research to be directed at specific contributions in isolation. Indeed the concept of the shell as a mediating rather than as merely a mineralised but porous boundary between the embryo and the nest/bulk environment is novel, it being introduced with a detailed discussion in 1982 (Board). This section contains those off-prints that contributed to the development of this concept.

Nevertheless an attempt has been made (Tullett & Board, 1977) to define control mechanisms that influence shell porosity. The question of the adaptive value, if any, of the various pore forms seen in avian eggshells is considered in Sections 3, 4 and 5.

* The collected works of Wilhelm von Nathusius on avian eggshells have been translated by C. Tyler (1964; The University: Reading).

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PROPERTIES OF AVIAN EGG SHELLS AND THEIR ADAPTIVE VALUE

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CONTENTS

I. INTRODUCTION

The advances made in the past decade in our knowledge of some of the properties and fine structure of eggshells have not been matched by field studies of eggs in the broad context of the breeding biology of birds. Indeed with the latter, the egg in the nest is often considered merely as an 'object' that needs heat, turning and protection. The controls that ensure embryo development are sought in the behaviour of the parents (White & Kinney, 1974). It is well known that through being porous the shell allows the embryo to exchange respiratory gases and that through being 'hard' it offers mechanical protection to the embryo. The purpose of this article is to discuss the proposition that eggshells have been adapted to meet the demands of the environment of the nest cup because parental behaviour alone cannot assure the well-being of the embryo.

In some instances the pressure of predation, availability of food etc., may influence the choice of nest site and nesting material such that a potentially inimical feature is imposed on the nest environment. Indeed the parents' adaptation to their own life style may also be in conflict with the eggs' requirements, for example with birds that have much preening oil on their plummage. Thus it is suggested that a high survival value is placed on adaptations of the shell that counter an inimical feature of the nest. As this contention has been discussed only occasionally, for example by Lack (1968) – the title of this article reflects that of his chapter – the present discussion must perforce be speculative, but it is anticipated that, by defining potentially inimical factors of the nest environment, those interested in

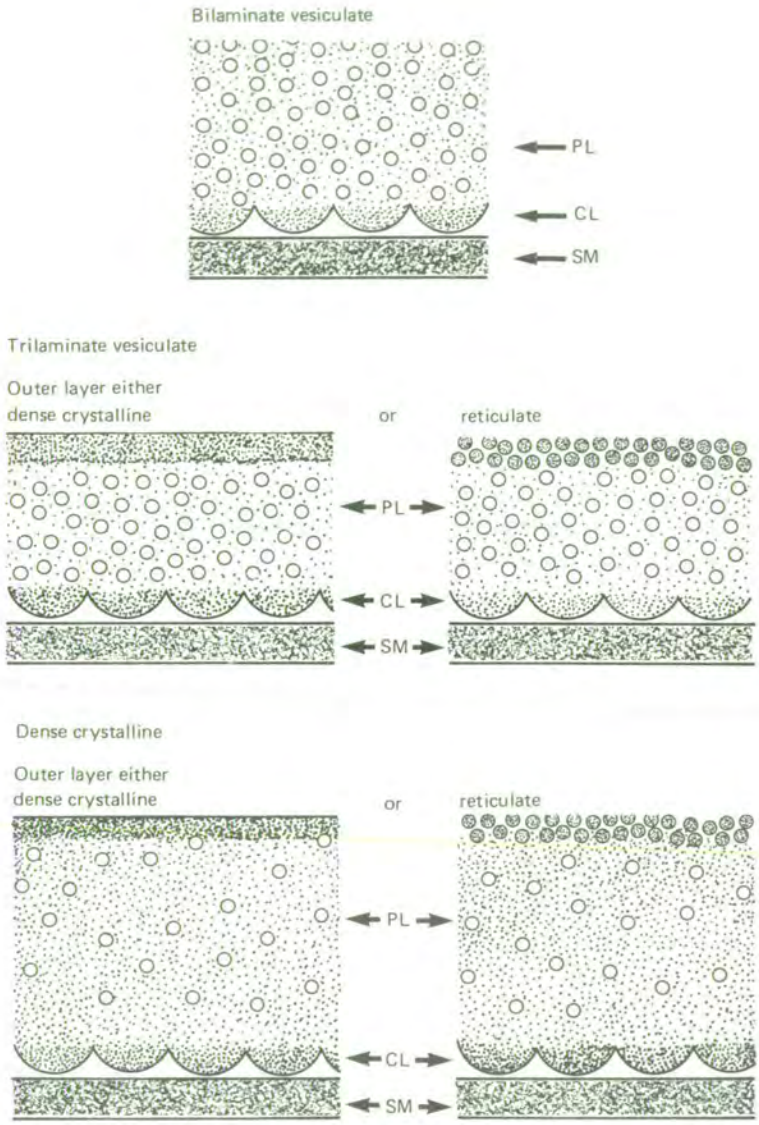

Fig. 1. An arbitrary classification of eggshells. PL, palisade layer; CL, cone layer; and SM, shell membranes.

the breeding biology of birds will extend their observations so that the means whereby such factors are ameliorated by parental behaviour, shell adaptations or the two in combination are defined.

II. EGGSHELL STRUCTURE

The information on eggshell structure supports three general conclusions: (1) the major component, calcium carbonate in the calcite form, is laid down on an organic matrix (Erben, 1970); (2) the cone layer (Fig. 1) is a feature common to

all eggshells (Tyler & Fowler, 1978), and (3) all shells contain pores that permit the diffusion of respiratory gases and water vapour (Ar & Rahn, 1980). Little is known in detail about the process of shell calcification (Wilbur & Simkiss, 1966) and argument persists about the detailed crystallography of the shell (Perrott, Scott & Board, 1980). As the information on shell formation has come from studies of the shell gland of the domestic hen (Mongin & Carter, 1977), we are not able to account for the diversity of the shell forms (Fig. 1) discussed in this article in terms of the physiology of this organ. Indeed the observations made in a recent survey of magnesium distribution across the shell suggests that there may well be differences in the secretory products of the shell glands of different classes of birds (Board & Love, 1980).

Although inadequate information precludes more than general observations on shell formation in birds, there is little doubt that all eggshells share a common genesis (Tyler & Fowler, 1978). Tufts of fibres on the outer surface of the shell membranes associated with round bodies, the mammillary cores, are the sites of shell initiation. With the domestic hen, tuft formation and calcification of the cores occurs in the red region, the distal 4 cm of the isthmus of the oviduct (Wyburn *et al.*, 1973). The fibres can be recovered by decalcification of the shell (Fig. 8C); the cores are features of fractured shells (Fig. 8D). As calcification proceeds, calcite grows towards and away from the shell membranes. The first-mentioned direction of growth results in crystals enmeshing the fibres linking the tufts and the shell membranes (Fig. 8B) and the second to the formation of entities having a roughly conical geometry (Fig. 8A), hence the name given to the inner surface of avian eggshells. In practice, however, there is a marked diversity in the geometry of these projections from the shell membranes, and systematic study might well show that their shapes are characteristic of major classes of birds.

As the cones continue to grow along radical axes, their edges meet (Fujii, 1974) and provide a base upon which gross calcification in the shell gland leads to the formation of the palisade layer (Fig. 1). It is generally agreed that this and the cone layer are the sole components of the true (calcitic) shell (Tyler, 1969*b*). The outer surface of the palisade layer may be overlaid, either completely or in part, by a stratum of small spheres (1–10 μm) which may be predominantly organic or inorganic in composition (Table 1). Such layers are often referred to as cuticle or cover (Tyler, 1969*b*) but there is perhaps merit in using a general term, shell accessory materials (Board & Scott, 1980), until more is known about their synthesis and functions.

In general large eggshells have thick palisade layers and *vice versa*. From studies of the eggshells of upwards of 150 species of birds, I have created an arbitrary classification which takes account of the microstructure of the palisade layer (Fig. 1). Two major categories of shell are recognized: (1) hard, brittle (snaps without evidence of bending) shells having a palisade layer (Fig. 9A) characterized by its dense crystalline structure and relative freedom from hollow spheres (vesicles), and (2) shells, commonly tough and flexible, whose palisade layer contains large numbers of vesicles (Fig. 9C). There are two sub-categories of (1): (*a*) the outer surface of the palisade layer is bounded by dense crystalline material

Table 1 Pore systems in avian eggshells*

Outer orifice of pore canal	Example
1. Open	Passion bird *Agapornis roseicollis* – Fig. 9D
2. Occluded with thin layer of amorphous material	Wedge-tailed shearwater *Puffinus pacificus chlorohyncus* – Fig. 10B
3. Plugged	
Plug – inorganic	Rhea *Rhea americana*
Plug – organic	Kiwi *Aeptyrex* spp. – Fig. 10A
4. Capped	
Capping material – organic spheres	Domestic hen *Gallus domesticus* – Fig. 9A
Inorganic spheres containing:	
Vaterite	
Amorphous calcium phosphate	Flamingo, e.g. *Phoenicopterus roseus*
5. Plugged/capped	
Pore orifice plugged with crystals and overlaid with bed of spheres containing Ca^{2+} and *P*	Mallee fowl *Leipoa ocellata* – Fig. 11
6. Reticulate	
Pore canal vents into a labyrinth of tubules in the shell below the outer surface	Stork, e.g. *Ciconia alba* – Fig. 9B

* For additional details see Board (1980); Board & Perrott (1979*a*, *b*) and Board, Tullett & Perrott (1977).

that contains even fewer vesicles than the major portion of this layer, and (*b*) the palisade layer terminates in a band of irregularly shaped crystals, the spaces between which interconnect to form a labyrinth of chambers around the shell (Fig. 9B). This has been referred to as the reticulate layer (Board & Tullett, 1975; Board, Tullett & Perrott, 1977).

The simplest vesiculate shell (Fig. 1) is essentially bilaminate, the dense crystalline cone layer merges with a palisade layer that contains many vesicles throughout its entire width (Fig. 9C). Indeed the fine structure of the shell's surface reflects the vesiculate nature of the palisade layer (Fig. 9D). Other vesiculate shells are essentially trilaminate because the outer surface of the palisade layer is formed from dense crystalline material, in which few vesicles occur, or a reticulate layer. As this arbitrary classification is based on a small sample (150 species) of birds, discussions of phylogenetic implications are unwarranted. The classification does permit, however, certain predictions to be made about the mechanical properties of a shell (see section III (3)) and thus should promote field studies of the mechanical properties of shells, and the nature, incidence and extent of insults to which they are exposed in the nest.

Not only is shell thickness related to egg mass (Ar, Rahn & Paganelli, 1979) but

also to the density of mammillary cores and hence cones (Tyler & Fowler, 1978). The latter relationship has been expressed thus (Tullett & Board, 1977):

$$\text{shell thickness } (\mu\text{m}) = 5948 \text{ cones}/0{\cdot}25 \text{ mm}^{2-0{\cdot}781}.$$

The number of cones per unit area decreases therefore as egg mass increases. Thus the circumference of the cones immediately before their edges meet is greater in large than it is in small eggs. As the cross sectional face of the cones has a fairly regular hexagonal shape (Tullett, 1975), all space would be filled if the mammillary cores were distributed uniformily. It has been deduced (Tullett, 1975; Tullett & Board, 1977) that irregular distribution is a prerequisite of pore formation, the canal starting at the gaps left between the edges of cones. As tufts of fibres are probably the determinants of the distribution of mammillary cores, the distribution of fibre-secreting cells in the oviduct will thereby influence initially such features as shell thickness and pore density. The actual spacing of the tufts of fibres will be influenced also by the albumen's absorption of 'plumping fluid' (Tullett, 1978). Absorption stretches the membranes, the extent of which will influence presumably the spaces between fibre tufts.

'Plumping fluid' has been associated also with the formation of the pore canal in the palisade layer (Tyler & Simkiss, 1959), its inward flow through gaps between cones ensuring that the canal is not occluded by crystal growth. It is difficult to imagine flow being maintained once fusion of the cones precludes stretching of the shell membranes. Perhaps it would be reasonable to suggest that once flow is prevented by the shell's rigidity, the diffusions along or concentration of ions (Mg^{2+}?) inhibitory to calcification in a static column of water would ensure that the canals remain open. This mechanism could also operate during the formation of the reticulate layer (Fig. 1). Whatever the mechanism(s) ensuring the formation of a pore canal, the result in the majority of eggshells is a tube having the geometry of a straight post horn running radially across the shell (Tyler, 1964, 1965, 1966, 1969*a*). In thick eggshells, such as those of the ratites (Tyler & Simkiss, 1959; Tyler & Fowler, 1979), the pore canal forks or branches extensively (Fig. 2). In eggshells having a reticulate layer (Fig. 1), the pore canal terminates at the bottom edge of this stratum, and the lumen of the canal vents to the void spaces in this layer.

In eggshells in which the pore canals traverse the entire width of the palisade layer, the external orifice can be: open (Fig. 9 D); plugged with organic material – e.g. tinamou, lily trotter (Board & Perrott, 1979*b*), and kiwi (Fig. 10 A) eggshells; occluded with amorphous material or overlaid with a stratum of spheres (Table 1). Differences in the morphology of the external pore canal was used to create an arbitrary classification of pore systems in eggshells (Board, 1980; Board *et al.*, 1977). As yet, however, the number of shells examined (those of *ca.* 150 species of bird) does not justify a phylogenetic analysis. It is notable, however, that within the ratites, considered by some to be monophyletic (Cracraft, 1973; Prager *et al.*, 1976), all but the covered and occluded systems of Board *et al.* (1977) occur throughout their geographical distribution (Figs. 2, 3). Although relatively few

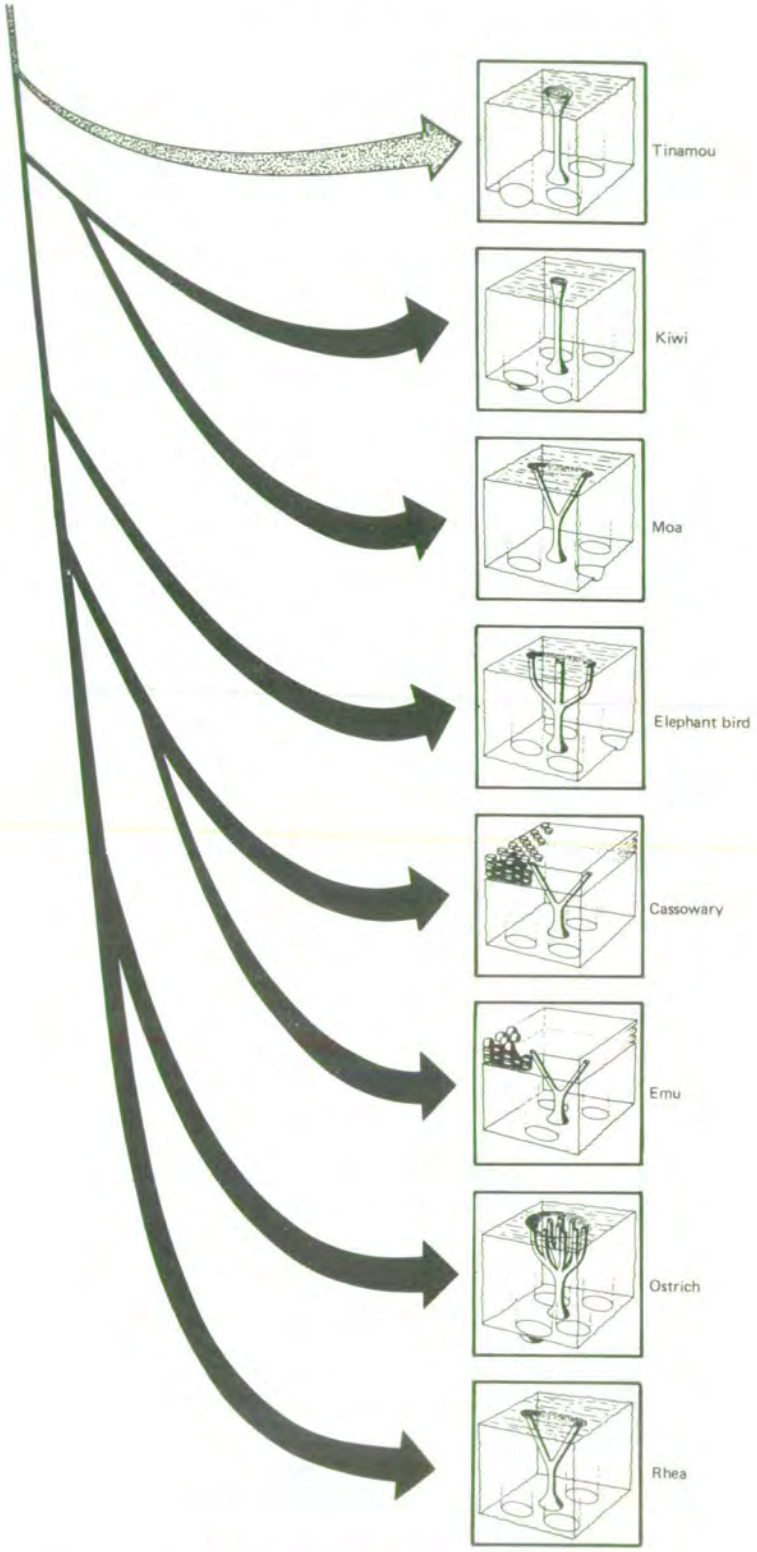

Fig. 2. For legend see opposite.

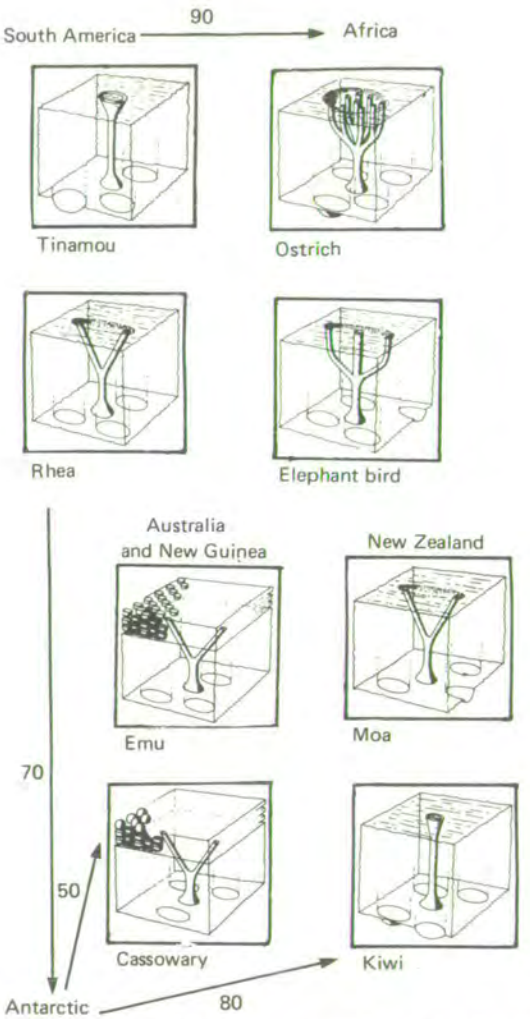

Fig. 3. The geographical distribution of the pore systems of the ratites listed in Fig. 2. The arrows accompanied by numerals are estimates of the millions of years since land masses were separated to such an extent that the dispersal of flightless birds was no longer possible. It is notable that the tinamous of South America, probably the site of origin of flightless birds, have pore systems similar to the kiwis, the outer pore orifice being plugged with organic material. The crystalline plug of the outer orifice of pores in the rhea eggshells can be regarded as a primitive or derivative form of the reticulate pore systems of the emus and cassowaries. The branching of the pore canals is a characteristic feature of the eggshells of the large ratites in all the continents shown.

Fig. 2. The pore systems in ratite eggshells set against the monophyletic relationship that is thought to obtain in this group. The outer orifices of the pores of tinamous and kiwis are plugged with organic material and those of the rheas with crystals. The cassowary and emu have reticulate shells. Branching of the pore canals, which is common to all the thick shells is most complex in those of the ostrich.

shells have been examined, the diversity of their pore systems does pose the question: are such adaptations one of the means whereby an egg is fitted to the nest environment? This topic is discussed in the next section.

III. EGGSHELL FUNCTIONS

The generally recognized contributions of the eggshell to the well-being of the embryo are: (1) conduction of respiratory gases, heat, light and sound; (2) conservation of water; (3) protection from mechanical damage; (4) provision of calcium for embryo bone formation; (5) adaptation to egg packing in nests and egg location in nesting sites, and (6) adoption of camouflage. Of these, 1–4 are common to all and 5 and 6 peculiar to some eggshells. Topics 1–4 have attracted much attention and the main observations of many investigations will be noted below. Tullett (1976) demonstrated that there were marked differences in the eggshells of different species of bird to conduct heat, but laboratory studies of this property, as well as the fine structure of the shell, would have to be considered in the context of the nest environment before the contribution of the insulative capacity of the shell to the well-being of the embryo could be assessed. Thus it would seem reasonable to expect a relatively sluggish heat transfer across a highly vesiculate shell. In practice this could have been an unavoidable consequence of evolution, the vesiculate eggshell being favoured by selection because of its mechanical properties alone. In other situations the density of vesicles in an eggshell may be associated with protection of the embryo from insolation; it is notable that the radial faces of cracked, highly vesiculate shells are brilliantly white due to the individual spheres causing total internal reflection of light. A similarly broad view needs to be taken when the adaptive significance of egg shape is considered. Although it is a commonly held view that the pear-shaped eggs of waders favours the close packing of four eggs in a nest, this may have been a fortuitous outcome of evolution because the characteristics of the parents' body, general shape and arrangement of the pelvic girdle, for example, may be the principal determinants of shape. The same heretical argument could be advanced also to account for the extreme pear-shape of Guillemot eggs. This form is considered to be a prerequisite of eggs brooded on narrow ledges because disturbance causes them to roll like a top rather than a marble. Indeed if egg shape is considered to have a high adaptive significance, then attention needs to be given to the control mechanisms that would have to operate in the oviduct immediately before shell deposition had provided a rigid, fixed shape. The list of functions given above tends to isolate one function from another; there is probably a subtle interplay between several of them such that natural selection has favoured a compromise between competing demands of the embryo in the egg or the embryo and the parent. To explain this viewpoint, it is petinent to consider some of the implications of birds having evolved a cleidoic egg.

(1) *Water conservation*

It is generally accepted that freedom from the need for exogenous water was the last major step in the evolution of the cleidoic egg (Needham, 1931). At oviposition a bird's egg contains sufficient water for the embryo's needs. As the latter still has a requirement for 'chemical communication' with the environment – the exchange of respiratory gases – the shell is porous and exchange occurs by diffusion (Paganelli, Ackerman & Rahn, 1978). As the oxygen molecule is larger than that of water, the pores allow evaporative water loss from the shell membranes, the extent of which is determined by the number of pores per shell, the thickness of the shell, time, temperature and the steepness of the diffusion gradient across the shell. Indeed evaporation is the major cause of loss from the egg's reservoir of water. In practice, however, depletion appears to be an obligate requirement so that an air cell of sufficient volume is formed to support pulmonary respiration of the embryo and to provide space for its movement during the hatching process (Rahn *et al.*, 1974) as well as to ensure that 'the water content of an egg at the end of incubation is to remain essentially the same as at the beginning' (Ar & Rahn, 1980).

A survey of the water vapour conductance of the eggshells of many species of birds has shown that the incubation period in days (I), egg mass in grams (M) and shell conductance (G_{H_2O}) are interrelated (Ar & Rahn, 1978):

$$I(G_{H_2O})/M = 5{\cdot}13 \pm 0{\cdot}86 \text{ mg/(g torr)}.$$

From a review of the literature, Drent (1970) deduced that on average eggs in nests lose about 16 % of the water present at oviposition, this amount presumably being optimal for incubation success. If shell conductance and incubation period are known then the diffusion gradient required to achieve a 16 % weight loss of an egg can be calculated. Indeed Rahn, Ackerman & Paganelli (1977) found a good agreement between the calculated diffusion gradient and that determined by measuring water vapour uptake in the nest by an egg hygrometer (an empty shell of species under study filled with dry silica gel). In practice the latter allows the determination of an average value; continuous monitoring of the relative humidity of nests (Howey, Board & Kear, 1977) has shown that the steepness of the diffusion gradient fluctuates markedly during the day, but the average is in accord with the predicted value. Other predictions based on the above formula have been examined. Thus with wet nests, such as those of coots, in which a shallow diffusion gradient would be expected, it has been demonstrated (Lomholt, 1976) that the shell's conductance is much greater than that of eggs of comparable size which are incubated in a dry environment. Likewise with eggs incubated for periods longer than would be predicted from their mass, the shells have a low conductance (Whittow, 1980), viz: the wedge-tailed shearwater *Puffinus pacificus chlororhynchus* (Ackerman *et al.*, 1980); fork-tailed storm petrel *Oceanodroma furcata* (Vleck & Kenagy, 1980); kiwi *Apteryx* spp. (Calder, 1978) and emu *Dromaius novae-hollandiae* (Vleck *et al.*, 1980). Moreover in some (barn swallow *Hirundo rustica*,

Packard *et al.*, 1977, and cliff swallow *Petrochelidon pyrrohonata*, Sotherland *et al.*, 1980) but not all eggs (black-billed magpie *Pica pica*, Taigen *et al.*, 1980) incubated at high altitudes (Carey, 1980), the shells' conductances are less than those that would be predicted from egg mass. Indeed it has been demonstrated that domestic hens kept at high altitude form shells whose conductances are lower than those laid at or near sea level (Rahn *et al.*, 1977). Thus there is much information to support the contention that shells are adapted to fit the egg to the environment so that optimal water loss is achieved during incubation. The corollary that the exchange of respiratory gases across the shell is subordinate to water conservation has been accepted in recent discussions (Vleck *et al.*, 1980; Sotherland *et al.*, 1980). A recent comparative study of the water vapour conductance of the eggs of the tunnel-nesting bank swallow *Riparia riparia* and those of the open nesting barn swallow *Hirundo rustica* led Birchard & Kilgore (1980) to conclude that the greater conductance of the former was an adaptation to meet the humid environment of a burrow.

A detailed analysis of the way in which oxygen consumption, water loss during incubaton, water vapour conductance of the shell and pore number of avian eggs relate to egg mass and incubation time led Rahn & Ar (1980) to propose: 'that all these functions are proportional to the product of egg mass and rate of development where the latter is defined as the inverse of incubation time', and to note 'these interrelationships account at the end of incubation for similar O_2 and CO_2 tensions in the air space of eggs, utilization of calories ($0{\cdot}5$ kcal $.g^{-1}$), and water loss ($0{\cdot}15 .g^{-1}$).

The contention that during the course of evolution birds have been able to adapt to a wide variety of nesting conditions by adjusting the thickness and porosity of their shells has been challenged by Simkiss (1980). He hatched chicks successfully even though 6 cm^3 of allantoic fluid had been removed about two-thirds the way through incubation or water loss accentuated by drilling holes in the broad pole of the shell. The chicks, though smaller than the controls, had a normal water content. He concluded that the growth of embryos is modulated according to the metabolites available and that the critical role of eggshell porosity had been over-emphasized. No doubt further studies will show that the shell's contribution to water conservation operates alongside metabolic control mechanisms of the embryo. As it is difficult to envisage the brooding parent 'sensing' the humidity of the nest, behaviour is likely to make a negligible contribution unless materials used for nest construction influence the humidity of the nest. Indeed there has been much speculation about the habit of many birds-of-prey who add green leaves to the nest cup throughout incubation and the possibility that this trait modified the nest humidity (I. Newton, personal communication).

As weight loss of eggs is the result of the diffusion of water vapour across the shell, Fick's law governing diffusion can be used to calculate the effective pore area of shells of known thickness. There has been one attempt only to compare this value and that obtained by multiplying the number of pores per shell by the average cross-sectional area of pores (Hoyt *et al.*, 1979). There was reasonable agreement

between the two values in spite of the technical difficulties involved with pore counts and, more especially, the determination of pore area. With counts, for example, dye penetration of the pore canals (Fromm, 1959) or acid etching of the shell so that the canals are enlarged (Tyler, 1953) probably gives an under and over-estimate, respectively, of pore numbers. Likewise the irregular outline of the inner orifice of the pore canal (Fig. 8B) poses problems when cross-sectional areas are being determined. In spite of these difficulties, the observations of Hoyt *et al.* (1979) support the view that the size of the inner orifice of the pore canal determines in part a shell's conductance. It is notable that recent studies of the small eggs of passerines have shown that a shell's conductance increases with incubation (Carey, 1979; Hanka *et al.*, 1979; Sotherland *et al.*, 1980). The factors contributing to this increase have not been defined but speculation (Sotherland *et al.*, 1980) has centred on a reduction of pore length or the opening of 'blind pores' by the embryo's absorption of Ca^{2+} from the shell. The latter proposition is not in agreement with the generally accepted view of pore formation (Tullett, 1975; Tullett & Board, 1977). Moreover any argument invoking the opening of 'blind pores' must consider also changes that may occur in the strength of a shell. Thus Bond (1980), who has adapted the model of Carter (1979), lays great emphasis on the rounded valley bottoms between cones; she contends that, should they become rough as a consequence of Ca^{2+} absorption, they would act as stress accentuation points and the shell would be weakened.

The control mechanisms operating in the isthmus/shell gland of birds that lay eggs adapted to wet nests, protracted incubation or brooding at high altitudes have not been identified, but Tullett (1978) is of the opinion that an interplay between the distribution of seeding sites for calcification and the albumen's uptake of 'plumping fluid' could result in a shell porosity that matches the dictates of the water vapour gradient obtaining across the eggshell in the nest.

(2) *Gaseous diffusion*

In this discussion of water vapour conductance of the shell, there has been no need to consider the overall geometry of the pore canals, merely their length and cross-sectional area. Indeed a notable feature of the allometric relationships discussed by Ar & Rahn (1978) is that they do not reflect the pore systems defined by Board (1980) and Board *et al.* (1977). Thus the method of determining the water vapour conductance of the shell – weight loss by an egg during storage with dry silica gel or some other desiccant at a controlled temperature – does not distinguish between shells having open, partially plugged or capped pore canals. If the pathways for water vapour conductance are considered as resistances in series (Fig. 4), then there are no demonstrable differences between shells having two (R_1 and R_{2a}), three (R_1, R_{2a} and R_{2b}) or four (R_1, R_2, R_3 and R_{3b}) resistances. This observation poses an important question: do shell accessory materials have any adaptive significance?

Lack (1968) was of the opinion that avian eggshells are adapted to fit an egg to its environment. Although he cited many examples of birds whose eggs might be

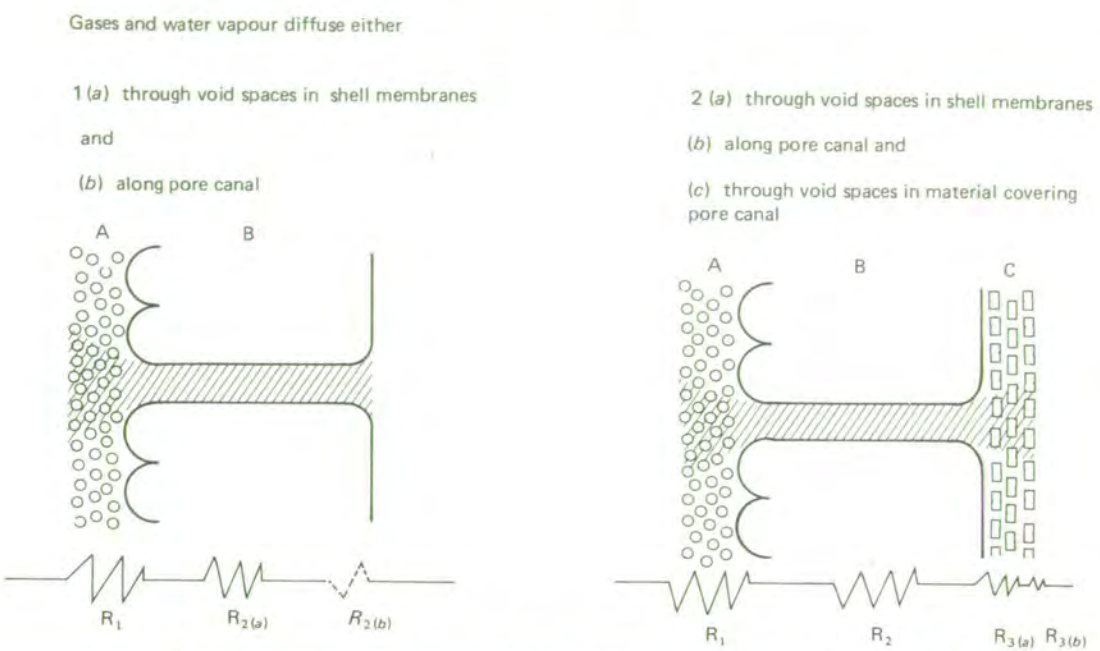

Fig. 4. The pore systems in avian eggshells depicted as resistance networks. The figure is derived from the observations of Board & Perrott (1979*a*, *b*) and Board, Tullett & Perrott (1977).

expected to need protection because the nests are wet or the parents bring water to the eggs on their plumage, he offered only general comments on the nature of the adaptations – the shell's surface was polished or powdery and thereby waterproofed. The importance of this property was not discussed. A tentative explanation for this need – and others – can be offered if certain implications of an egg achieving the cleidoic state are considered. Although independence of a requirement for exogenous water goes some way in 'isolating' an embryo from its environment, the need to exchange respiratory gases calls for a porous shell. It would seem reasonable to assume that exchange must not be stopped even temporarily or the shell's potential to conduct gases diminished so that its ultimate capacity was insufficient to meet the embryo's demands late in incubation. Flooding of the pores would stop diffusion and this may be the situation Lack (1968) was considering when he identified waterproofing as an important property of an eggshell. Even the flooding of a few pores may be important because microbial infection of the egg's contents is dependant upon the translocation of bacteria along water-filled canals (Board & Fuller, 1974). On *a priori* reasoning several conditions could lead to a diminution of the conductance of eggshells: the deposition of mud, preening oils and nest debris on the outer surface of the shell; attrition resulting in pores being blocked by dirt, or microbial growth, particularly that of moulds (Board *et al.*, 1979), clothing the outer surface of the shell. The influence of some of these on water loss from the eggs of domestic hens is shown in Fig. 5. If the

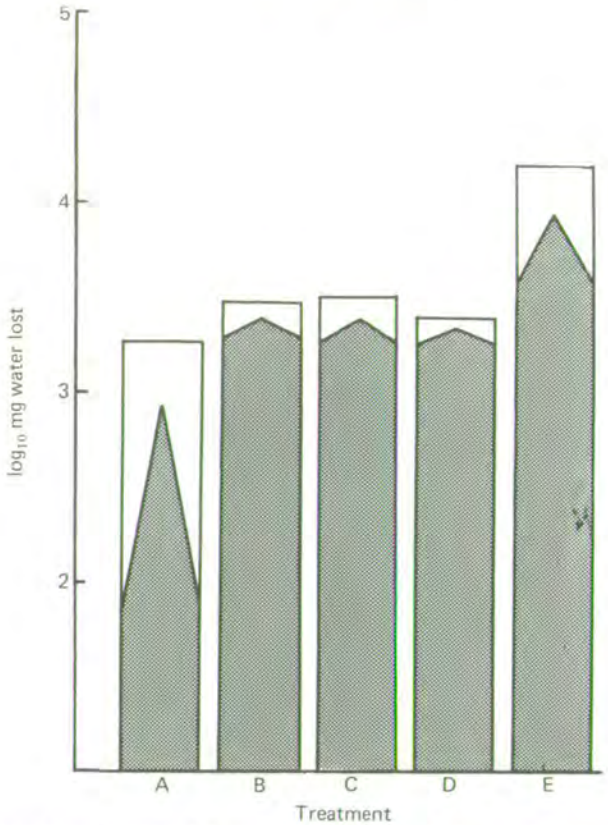

Fig. 5. Water loss from the eggs of domestic hens. Freshly laid eggs of domestic hens were stored in a microbiological incubator at 37 °C. (A) Oiled eggs – the shell was wiped with a paper tissue impregnated with light petroleum jelly; (B) polished shells – the eggs were polished with glass paper (Grade oo, flour paper): (C) cuticle-less eggs – eggshells were immersed in ethylene-diaminetetra-acetic acid (5 %, w/v, aqueous solution, pH 7·5) and the cuticle removed with paper tissue while the egg was held under running tap water; (D) control eggs, and (E) cracked shells – the eggs were rapped sharply on the edge of the bench. Twelve eggs in each treatment; apex of pointed column, average of the observations, and unshaded area, range of observations. (My unpublished observations).

pore systems in eggshells (Fig. 4) are considered as resistance networks, then certain of the resistances can be identified tentatively as the means whereby eggshells are adapted to counter some of the problems listed above.

Waterproofing is a relatively vague term and it is preferable (Board, 1974) to consider two properties of an eggshell: water repellency – resistance of the pores to flooding when no hydraulic loading obtains; and water resistance – the prevention or, at best, impediment of pore flooding in the presence of a hydraulic load. As the washing of hens' eggs intended for human consumption can result in a high incidence of rotting, the poultry industry has done much work on factors that cause pores to be flooded, the first stage in the rotting process (Board & Halls, 1973).

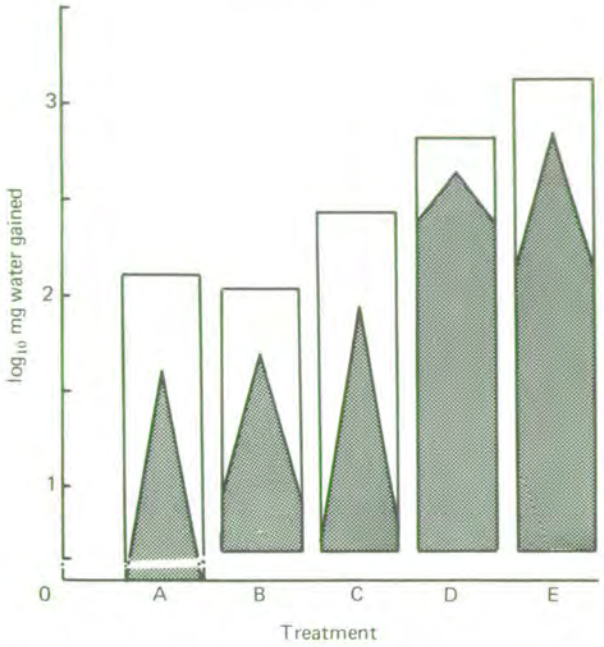

Fig. 6. Water uptake by the eggs of domestic hens. Eggs at 37 °C were immersed for 5 min. in water containing ice, dried and weighed. (A) Oiled eggs; (B) polished eggshells; (C) control; (D) cuticle-less eggshells, and (E) broken eggshells. The treatment of eggs and explanation of the columns are given in Fig. 5. (My unpublished observations).

From a review of the literature, Board (1980) presented evidence that work needs to be done if liquids having viscosities greater than 30 dynes are to be introduced into the pore canal. Sufficient work can be done by: (1) allowing a warm egg to contract in cold water – the small pressure differential caused by the unequal contraction of the shell and egg contents causes water to be sucked into the pore canals; (2) imposing a pressure greater than the atmospheric on water covering eggs in a sealed container, and (3) drawing and suddenly releasing a vacuum on eggs submerged in water. If change in weight is used as an index of the efficacy of these methods, then it can be demonstrated that the shell accessory material (the cuticle) plays an important part in the water resistance of the eggshells of domestic hens (Fig. 6). In terms of a resistance network, the glycoprotein spheres of the cuticle impose the Resistance R3a of Fig. 3. This resistance was not overcome (Board, 1974) when water drops from the rose of a horticultural watering can were allowed to fall 2 metres upon the domestic hens' egg. Such treatment flooded the open pores in those of the pigeon *Columba* spp. These observations suggest that studies of the breeding biology of birds that lay eggs with open pores should attempt to identify the strategy that prevents flooding of the pore canal. Is flooding prevented by: (1) parental behaviour when the eggs are laid in open nests, (2) nest design, (3) nest site or (4) breeding during the dry season? It is

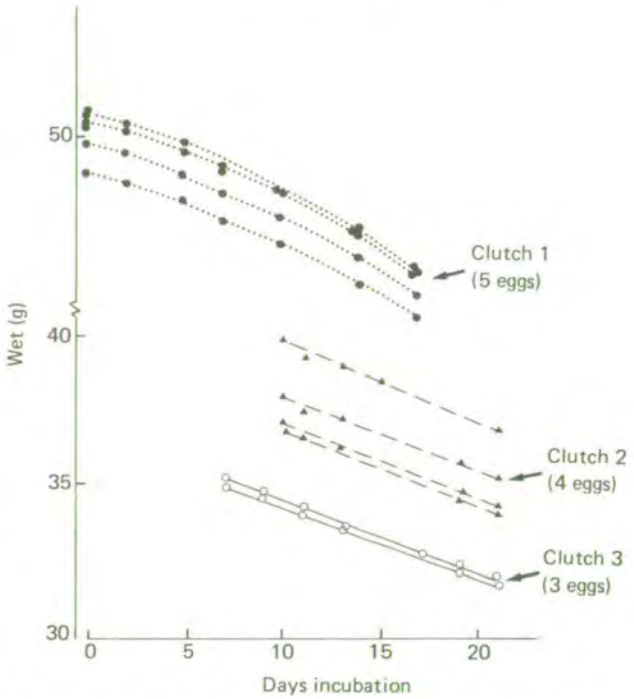

Fig. 7. Water loss from the eggs of coots. The nests of coots at the Slimbridge Wildfowl Trust or the adjoining Frampton gravel pits were visited regularly (by canoe when necessary); the eggs were taken to the bank, marked with pencil, weighed and returned to the nest. Weighing was continued until hatching or predation occurred. (Karl Lane's and my unpublished observations.)

noteworthy, for example, that the burrow-nesting wedge-tailed shearwater (*Puffinus pacificus chlororhynchus*) lays eggs with open pores (Fig. 10B).

The corollary to the argument discussed above is that eggs laid in wet nests or in open nests during a rainy season ought to have pores that are partially plugged, capped with shell accessory material or overlaid with the reticulate layer. Although this proposition has been discussed (Board *et al.*, 1977), field studies would have to attempt to distinguish between adaptations that render a shell water resistant and those that protect pores from blockage with extraneous material. Indeed the latter adaptations may be of greater importance because a potentially inimical feature, for example, mud, could be a persistent, but rain an occasional, feature of the nest environment. In the context of the shell as a resistance network, attention needs to be given to R3a and b (Fig. 4) as they are the possible means whereby the shell's potential to allow diffusion is maintained throughout incubation. The saturated platform of vegetation upon which grebes (Podicipitiformes) incubate their eggs would appear to provide a particularly unfavourable environment to the shell. It is well known that the shells become heavily stained and polished during incubation. The water conductance of coots' eggshells is unchanged during incubation in a nest not dissimilar to that of grebes (Fig. 7). The calcite shell of

Figs 8–11. The specimens were sputter coated *in vacuo* with gold and examined with a JEOL 35c at an accelerating voltage of 15 kV.*

Fig. 8. (A) Scanning electron micrograph (SEM) of the inner surface of a megapode eggshell which had been freed of shell membranes by boiling in 5·0% (w/v) aqueous sodium hydroxide. T, tip of cones showing tracks left by fibres of the shell membranes. O, inner orifice of pore canal. Bar marker = 100 μm.

(B) SEM of radial face of a peice of snapped eggshell of domestic hen. F, fissures in the cuticle (C) overlying the outer surface of the shell (S). The origin of the pore canal (PC) is at the junction of cones (CO) the tips of which are attached by fibres (FI) to the shell membranes (SM). Bar marker = 100 μm.

(C) SEM of a plastic model of the inner surface of the eggshell of the domestic hen. A piece of eggshell was freed of shell membranes by boiling in 5·0% (w/v) aqueous sodium hydroxide, washed in running tap water and, when dry, its inner surface painted with nail varnish containing Nylon. The mineral portion of the shell was dissolved in concentrated nitric acid. The plastic produced a cast of the valleys between the cones (shown in A) and provided a framework in which the fibres (F) within the tips of the cones were retained. Bar marker = 20 μm.

(D) SEM of a tangenital section of the cone of the eggshell of the passion bird *Agapornis roseicollis*. Crystals (C) radiate out from the core (CO) of the cone (CN) which rests on the fibrous shell membranes (SM). Bar marker = 10 μm.

Fig. 9(A) SEM of the outer part of radial face of the snapped eggshell of a domestic hen. The cuticle (C) overlie the outer orifice of the pore canal (PC) and a stratum of dense crystalline material (CM). The major portion of the shell, the pallisade layer (PL), contains few vesicles (V). Bar marker = 10 μm.

(B) SEM of the radial face of a snapped piece of eggshell of the white stork *Ciconia alba*. The pore canal (PC), which originates at the junction of cones (C) attached to the shell membranes (SM), retains its identity for about half of the width of the shell. It then merges with a labyrinth of caverns (CA) into which finger-shaped crystals intrude. Bar marker = 100 μm.

(C) SEM of the radial face of a snapped piece of eggshell of a passion bird *Agapornis roseicollis*. The shell is notable for the dense crystalline cone layer (CL) and the large number of vesicles (V) distributed throughout its entire width. Bar marker = 10 μm.

(D) SEM of the outer surface of the eggshell of a passion bird *Agapornis roseicollis*. The vesicles (V) and open orifice of the pore canal (PC) are notable features. Bar marker = 10 μm.

Fig. 10(A) SEM of the outer surface of the eggshell of a kiwi *Apteryx* sp. The outer orifice of the pore (P) is plugged (PC) with an aggregate of spheres. Bar marker = 20 μm.

(B) SEM of the outer surface of an eggshell of a wedge-tailed shearwater *Puffinus pacificus chlororhyncus*. Vesicles (V) and amorphous material (AM) encircling the pore orifice are notable features. Bar marker = 20 μm.

(C) SEM of the outer surface of an un-incubated eggshell of the helmeted guinea fowl *Numidia meleagris*. The surface is fissured (F) extensively. Bar marker = 100 μm.

(D) SEM of the outer surface of an incubated eggshell of the helmeted guinea fowl. The egg had been incubated by the parents in a scrape in the ground. The surface of the shell was polished and the few fissures (F) were associated with cuticle overlying the pore canals. Bar marker = 100 μm.

Fig. 11 (A) SEM of the radial face of a piece of snapped eggshell of the mallee fowl *Leipoa ocellata*. A lanceolate pore canal (PC) is plugged with crystalline material (CM) and overlaid with a stratum of spheres (S) containing amorphous calcium phosphate. Bar marker = 50 μm.

(B) SEM showing the plug (P) in the outer orifice of the pore canal (PC) in an eggshell of a mallee fowl. The fissures (F) at the outer surface of the plug are notable features. Bar marker = 60 μm.

(C) SEM of radial face of a piece of snapped eggshell of the jungle fowl *Megapodius freycinet*. The outer orifice of the pore canal (PC) is plugged with spheres (P). A similar arrangement was noted with the eggshell of the brush turkey *Alectura lathani*. Bar marker = 100 μm.

(D) SEM of the spheres in the cover on the outer surface of an eggshell of the jungle fowl *Megapodius freycinet*. The spheres are rich in amorphous calcium phosphate. Bar marker = 2 μm.

* Specimens were kindly donated by: Dr R. S. Seymour (Adelaide University, Australia): Fig 8(A); Dr G. Bartholomew (UCLA, USA): Fig. 8(D); Dr J. Parsons (Kenya): Fig. 9(B); Trustees of the British Museum and Dr R. A. Ackerman (Scripps Institute, USA): Fig. 10(A). The helmeted guinea fowl nested in the garden of the Author. Thanks are due to the Science Research Council for provision of electron optic equipment.

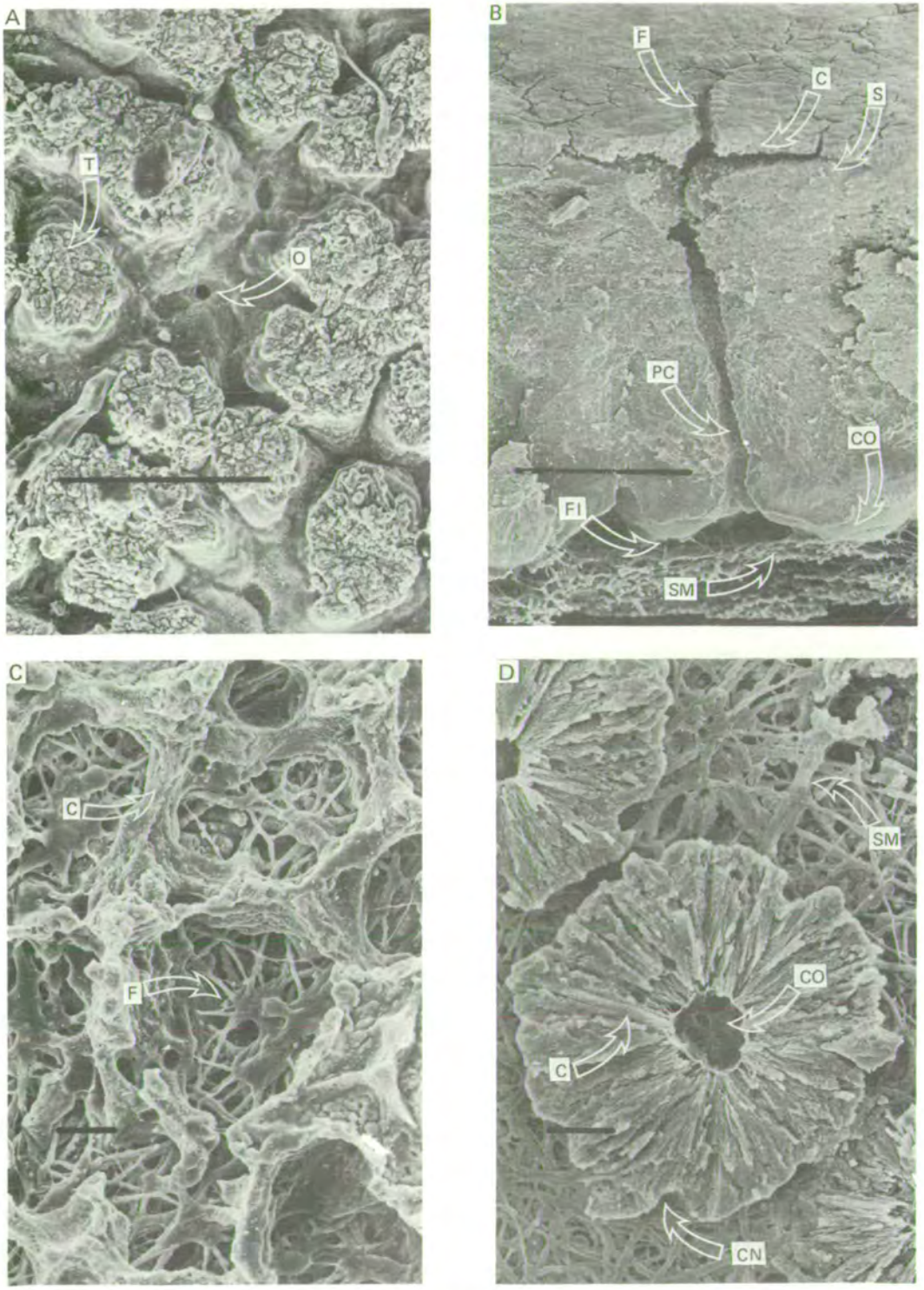

Fig. 8.

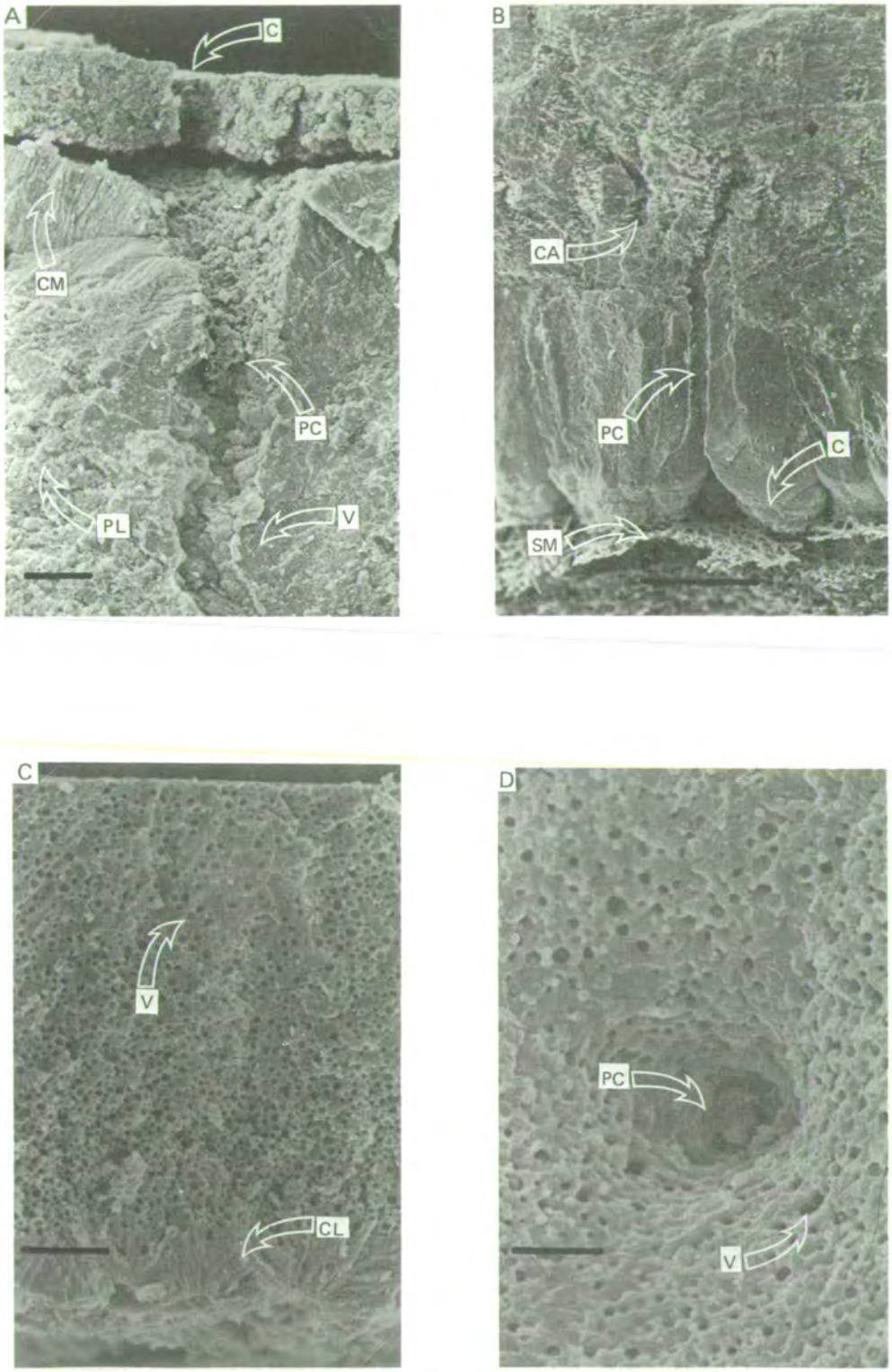

Fig. 9

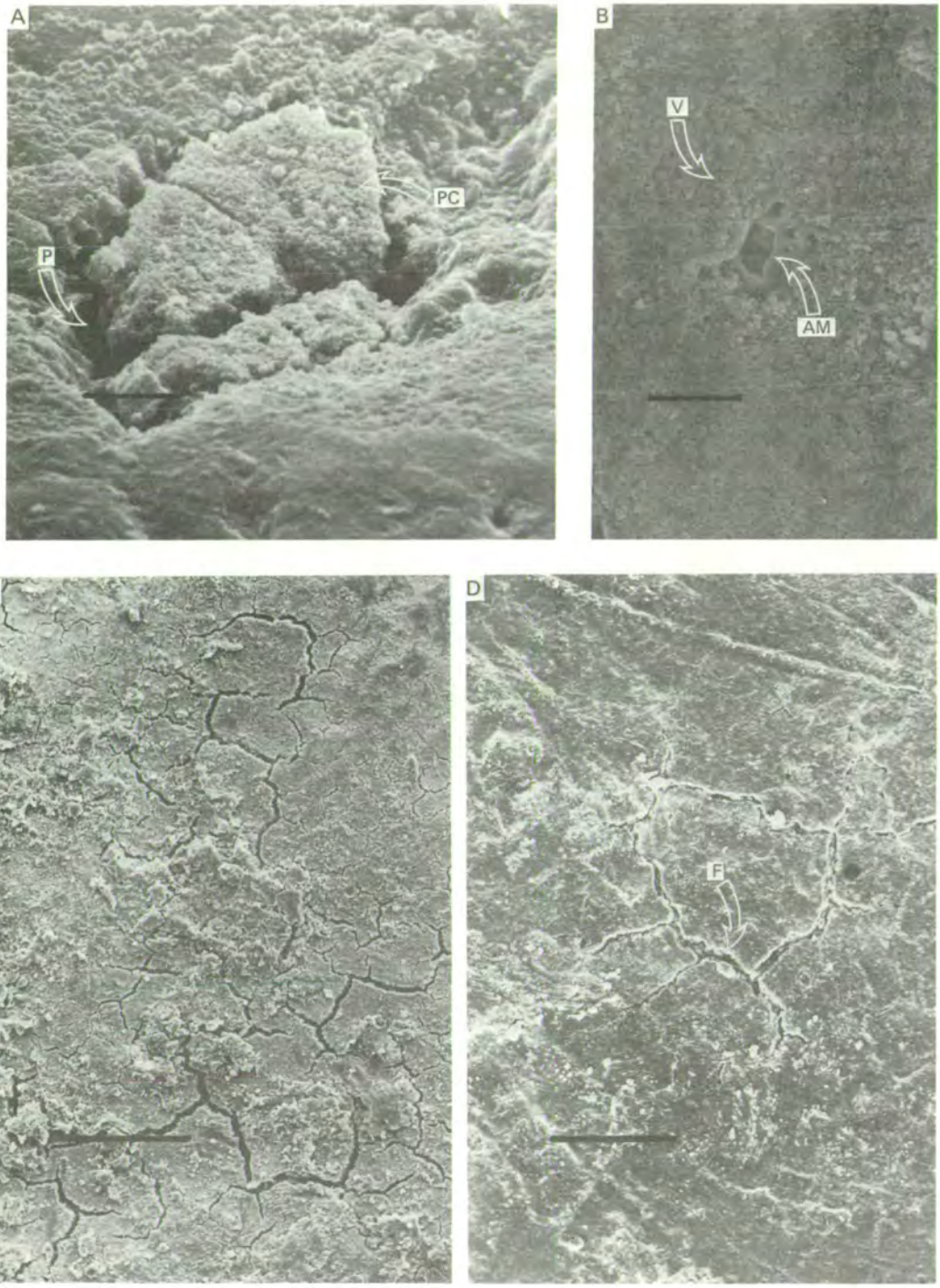

Fig. 10

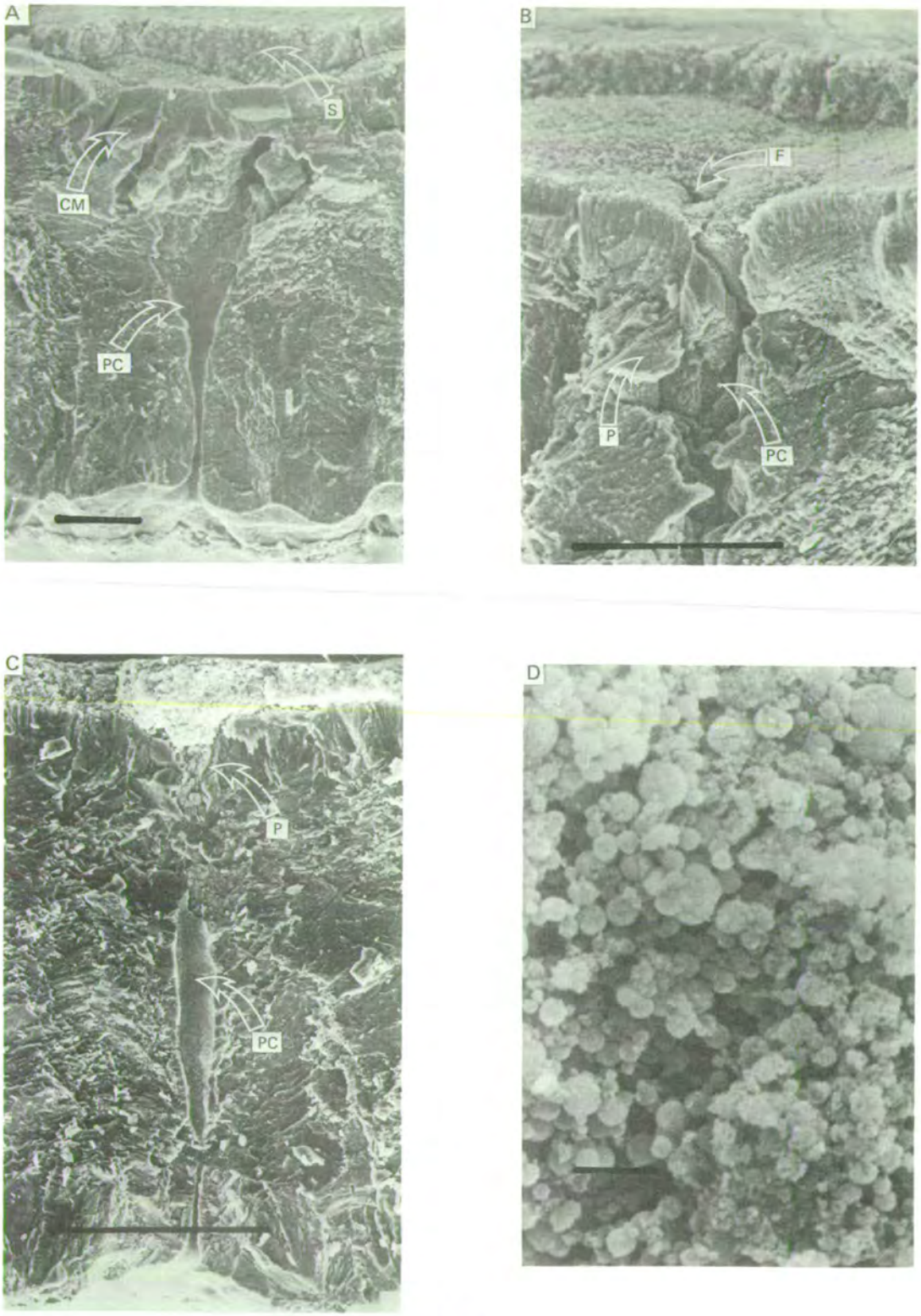

Fig. 11

grebe eggs is covered with a layer of spheres and the outer surface of the layer is bounded by amorphous material containing many fissures (Board *et al.*, 1981). Thus the lumen of the individual pores in the calcitic shell vents to the interconnected void spaces between the spheres and the latter vent via the fissures to the nest atmosphere. As the fissures are evident in stained as well as unstained shells, there must be a mechanism that stops their blockage with debris. Could it be that by turning the eggs, the fissures are kept open – some debris being rubbed off by the parents' feathers and some being pushed through the fissures? It is notable that the spheres in incubated eggs have a coating of extraneous material. These observations suggest that some feature of the grebe's behaviour on the nest maintains resistances R3b (Fig. 4) at an optimal value and that, although debris may increase the value of resistance R3a, the attained value never becomes a serious impediment to the shell's conductance. The eggshells of the helmeted guinea fowl *Numidia meleagris* are polished during incubation in the wild or in a nest box with a broody hen (Board & Perrott, 1981). At oviposition the undulating surface of the shell is covered with a fissured layer of spheres, but it acquires an amorphous appearance with incubation, the only characteristic feature being the fissures in the plug of spheres in the external orifice of the pore canals (Fig. 10C, D). Not only do the spheres in the pore plugs acquire nest debris during incubation, but those on the tips of the ridges are rubbed off. Thus the ridged surface of the shell protects the pore plug from attrition; the fissures in the latter permit the exchange of respiratory gases. With eggshells covered with a deep bed of spheres it could well be that nest debris infiltrates the outer layer of spheres thereby increasing the value of Resistance R3b (Fig. 4), but serious impediment to gaseous diffusion does not result because diffusion is forced to occur across the entire surface of the shell rather than via the small sector of spheres overlying each individual pore. In this context it is worth quoting from Tyler's (1969*a*) paper on eggshells having an outer covering of spheres: "When dye is painted on the side of a piece of shell which has been treated with alkali, the dye moves up the pore channels and then spreads out over the cover to give large circles of dye with a dark centre. These circles ultimately coalesce and cover the whole of the surface with dye."

The removal of spheres and debris from the outer surface of the egg would be another strategem that could theoretically maintain Resistance R3b (Fig. 4) at an acceptable value.

Following the theme of Lack's (1968) discussion that eggs incubated in wet nests need to be waterproofed, Table 2 contains a list of the shell adaptations found in eggs laid in wet nests. It is notable that all the eggs have either a cover of shell accessory materials over the entire shell surface or plugs in the outer pore orifice. In addition, the Table lists eggs laid in very exposed nests; again adaptations of the shells are a notable feature. Although there seems to be much circumstantial evidence linking shell adaptations with open or wet nests, there is a need for field observations to test the hypothesis that these adaptations waterproof the eggs or in some way or another prevent the impedance of gaseous diffusion. The removal

Table 2. *Pore systems and nest types*

Pore system	Nest type
Outer orifice open	A crude bundle of twigs: pigeons *Columba* spp.*
Outer orifice of pore contains a plug of material rich in sulphur	A platform of vegetation on surface of water: the lily-trotter *Micropara capensis*†
Outer orifice of pore covered with:	
(*a*) Spheres of glycoproteins	A ground scrape, the domestic hen‡
(*b*) Spheres of vaterite	A cup containing vegetation, non-parasitic cuckoo *Crotophagi ani*. A platform of rotting vegetation, the blue-eyed shag§
(*c*) Spheres rich in phosphorus and calcium	A platform of vegetation on surface of water, the grebes‖ A mud cone at the edge of soda lakes, the flamingoes** A mound of sand or fermenting vegetation, the megapodes.**

* Board, Tullett & Perrott (1977)
† Board & Perrott (1979*a*)
‡ Board & Halls (1973)
§ Board & Perrott (1979*b*)
‖ Board (unpublished observations)
** Board (unpublished observations; see Fig. 11).

of shell accessory materials from eggs would be one practical approach in field studies. Another would be to take the eggs with one type of pore system and put them in the nest with eggs having another and study weight loss and changes in the fine structure of the shell's surface. The non-destructive technique for studying the structure of shell surfaces (Board, 1981) would probably be helpful in such work. Perhaps the best experimental approach would be to use dummy eggs of the size and shape of the species' under study. With careful design and production it ought to be feasible to produce an egg with which to determine: (i) impairment of diffusive capacity with incubation, and (ii) the amount and probably the nature of debris acquired during incubation.

So far in this discussion, emphasis has been given to plugged or covered pores. Although the reticulate shell is fairly widely distributed in the bird world, there is no obvious link between this shell type and the nest environment.

It was noted above that mould growth on eggshell could pose a problem in that it could be expected to increase the values of resistances R3a and b (Fig. 4). The experience of the poultry industry that the glycoprotein cuticle on the shells of domestic hens are colonized by bacteria when stored under humid conditions (Board *et al.*, 1979) suggests that such eggs are poorly adapted to incubation in wet nests or for protracted periods in humid conditions, such as would be expected to obtain in a burrow. Moreover a recent study (Board *et al.*, 1979) has shown that bacterial colonization of the shell of the domestic hens' egg can result in the digestion of the glycoproteins by proteolytic pseudomonads. Thus the wetness or humidity of nests may have had an important selective role in the evolution of the shell accessory materials listed in Table 1. Indeed the eggs of the megapodes that

incubate their eggs in mounds of sand or fermenting vegetation are of particular interest in this context. A recent study (unpublished observations) has shown that the outer surface of the shells of three species of megapodes (Table 2) are covered with a thick stratum of spheres which are rich in phosphorus and calcium and which are not removed by boiling in sodium hydroxide (5 %, w/v, in water). The spheres form a cover over the outer orifice of the pore canal which in one species, the mallee fowl *Leipoa ocellata*, is plugged by large crystal aggregates (Fig. 11 A, B, C, D). It is notable also that calcium carbonate in the vaterite form is the accessory material on shells of non-parasitic cuckoos that lay their eggs in nests containing freshly collected vegetation (Board & Perrott, 1979*b*) and those of shags, etc., that lay their eggs on decomposing vegetation (Tullett *et al.*, 1976; Board & Perrott, 1976*b*).

(3) *Mechanical protection*

As eggshells are 'hard' it is a commonly held belief that they play an important role in protecting the embryo from mechanical damage, an external 'skeletal' support in the words of Ar *et al.* (1979). They recognized that the shell must be strong enough to support the mass of the incubating bird and that of the egg without being so strong that hatching would be impeded. These authors obtained a linear relationship by regressing yield point force (the load in grams required to initiate crushing when force was applied to both poles of an egg) against the initial mass (in grams) of the egg and concluded that a safety factor is built into eggshells such that they are not flattened by the brooding parent. Studies of the force needed to continue crack propagation after the initial fracture of the eggshell have revealed (Bond, 1980) that shells are either hard and brittle or tough and flexible and that the chick's hatching technique is adapted accordingly. Thus chicks in hard brittle shells need to make only a few holes in the shell before pushing off the broad pole of the shell, whereas those in tough flexible ones need to make many holes around a latitude of the shell before attempting to escape.

It is evident from Figs. 5 and 6 that water loss and water absorption are accentuated when the shell is fractured and the resistance networks (Fig. 4) are damaged. A fractured shell offers little resistance to bacterial invasion of the egg contents (Baker, 1974). Thus the mechanical properties that protect the integrity of the shell may be of equal or greater importance than those protecting the embryo from crushing. Indeed the properties needed to resist crushing may differ significantly from those that prevent cracking; the former has to resist quasi-static and the latter shock loading. It is well known that a wine glass will support a heavy weight providing all interfaces are perfectly smooth but it is shattered by a sharp blow. Thus tests of shell strength by imposing quasi-static loads will not necessarily indicate the shell's resilience to damage by collision with another egg or some hard object in the nest. Indeed the linear relationship of force and egg mass noted by Ar *et al.* (1979) when using quasi-static loading obscures the two types of shell discussed on p. 3 or the shell types indicated in Fig. 1. If the latter are considered for resistance to damage by impact, then the theoretical order of resistance would be: bilaminate vesiculate > trilaminate vesiculate > dense crystalline.

A shell's resistance to damage by impact may be accentuated by shell accessory materials. Thus Tyler (1969*c*) noted that it took hundreds of impacts by a ball-bearing to break the shell covered with a stratum of spheres but 10-fold fewer if the shells were freed of spheres. Erosion resistance is probably another important property of eggshells in certain types of nest where there is the potential for pores to be blocked by attrition. In this situation the shell types of Fig. 1 would rank: dense crystalline and trilaminate > bilaminate vesiculate. This order could of course be changed by shell accessory materials.

Although attention has been focussed on thin fragile eggshells as the cause of the catastrophic collapse of populations of raptors following the agricultural use of D.D.E. (1, 1 dichloro-2, 2-bis-(*p*-chlorophenyl)ethylene) (Ratcliffe, 1970), systematic studies do not appear to have taken into account the three stress factors – impact, compression and erosion – discussed above. Indeed, although shell strength has long been assumed to be a vital factor in the successful breeding of birds, no attempts have been made to define the means whereby a shell is endowed with the properties appropriate to a particular type of nest or brooding behaviour. Empirical observations suggest that structure and environment may well by linked. Thus the eggshell of the wedge-tailed shearwater is tough and flexible, of the trilaminate vesiculate type, whereas eggs of the domestic hen, which are of comparable mass, have hard-brittle shells of the dense crystalline type (Fig. 1). The former lays one egg in a burrow whereas the latter produces a clutch of 12 or so eggs in an open nest where there must be a high incidence of egg-to-egg collisions. I have noticed, for example, that the guinea fowl *Numidia meleagris* takes off explosively when disturbed on the nest and the eggs are scattered beyond the boundary of the nest cup. It would seem reasonable to assume that the exceptional strength of these eggshells may have been selected to counter shell damage when the hen is escaping from a predator. Likewise the nest cup of the oyster catcher *Haemalopus ostralegus* – a scrape in the ground containing stones – would appear to offer a hostile environment to an egg, its shell needing to resist damage by impact, compression and erosion. As the shell is of the trilaminate vesiculate type, it would appear to be well adapted to resist erosion and fracture by impact, the two most probable inimical factors to which it is exposed throughout incubation.

Shell strength, particularly of eggs laid in open nests, needs to be considered in the context of predation by other birds also. As most avian predators break the shell by pecking – a good example of repeated shock loading of a point on an eggshell – the strength of the shell will presumably dictate the eggs that are vulnerable to a particular predator. For example, starlings are able to break tern eggs but not the 'harder' gull eggs (I. Newton, personal communication). Indeed the strength of an eggshell may be of such a magnitude that avian predators cannot break the shell with their beaks. This is the case with the eggshell of the ostrich *Struthio camelus*, and the principal predator, the Egyptian vulture *Neophora percnopterus*, breaks the shell by dropping stones on it. On casual contemplation the shiny, white-cream ostrich eggshell would appear to be an incongruous adaptation to open nests that are left unattended before the onset of incubation.

Indeed Bertram & Burger (1981) were able to reduce the rate of predation by staining the shells with a brown pigment. When treated and untreated eggs were exposed to the sun, the internal temperature attained in the former was greater than that of the latter. Thus evolution appears to have favoured a compromise such that the rate of predation of eggs rendered conspicuous by colour is offset by the colour protecting the blastoderm from damage by heat.

These isolated observations suggest that without considering the type of nest and parental behaviour there is no simple answer to the question: what is the minimum egg mass that can be contained in a hard, brittle shell that has sufficient resilience to withstand cracking but is sufficiently weak to allow hatching?

IV. SUMMARY

1. An arbitrary classification of avian eggshells is proposed.

2. The role of the eggshell in conserving the water in eggs at oviposition is discussed. There is as yet no correlation between this property and the pore systems in avian eggshells.

3. The pore systems may act as diffusion pathways and the hypothesis has been advanced that in many eggs the shells are adapted so that restriction of gaseous diffusion by mud, preening oils and nest debris is prevented.

4. The mechanical properties of the shell are considered in the novel context of defence against (*a*) attrition that could lead to the pores being blocked with dust, and (*b*) cracking that would destroy the diffusion pathways noted in 3.

5. The overall objective of the review was to discuss the concept that avian eggshell are adapted to fit an egg to the nest environment.

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VI. REFERENCES

Ackerman, R. A., Whittow, G. C., Paganelli, C. V. & Pettit, T. N. (1980). Oxygen consumption, gas exchange and growth of embryonic Wedge-tailed shearwaters (*Puffinus pacificus chlororhynchus*). *Physiological Zoology* **53**, 210–221.

Ar, A. & Rahn, H. (1978). Interdependence of gas conductance, incubation length and weight of the avian egg. In *Respiratory Function in Birds, Adult and Embryonic*. (ed. J. Piiper), pp. 227–238. Springer-Verlag: Berlin.

Ar, A. & Rahn, H. (1980). Water in the avian egg: overall budget of incubation. *American Zoologist* **20**, 373–384.

Ar, A., Rahn, H. & Paganelli, C. V. (1979). The avian egg: mass and strength. *Condor* **81**, 331–337.

Baker, R. C. (1974). Microbiology of eggs. *Journal of Milk and Food Technology* **37**, 265–268.

Bertram, B. C. R. & Burger, A. E. (1981). Are ostrich *Struthio camelus* eggs the wrong colour? *Ibis* **123**, 207–210.

BIRCHARD, G. F. & KILGORE, D. L. (1980). Conductance of water vapour in eggs of burrowing and non-burrowing birds: implications for embryonic gas exchange. *Physiological Zoology* **53**, 284–292.

BOARD, R. G. (1974). Microstructure, water resistance and water repellency of the pigeon eggshell. *British Poultry Science* **15**, 415–419.

BOARD, R. G. (1980). The avian eggshell – a resistance network. *Journal of Applied Bacteriology* **48**, 303–313.

BOARD, R. G. (1981). A non-destructive method for studying the fine structure of avian eggshell. *British Poultry Science* **22**, 407–408.

BOARD, R. G. & FULLER, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biological Reviews* **49**, 15–49.

BOARD, R. G. & HALLS, W. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science* **14**, 69–97.

BOARD, R. G. & LOVE, G. (1980). Magnesium distribution in avian eggshells. *Comparative Biochemistry and Physiology* **66A**, 667–672.

BOARD, R. G. & PERROTT, H. R. (1979*a*). Vaterite, a constituent of the eggshells of the non-parasitic cuckoos, *Guira guira* and *Crotophagi ani*. *Calcified Tissue International* **29**, 63–69.

BOARD, R. G. & PERROTT, H. R. (1979*b*). The plugged pores of the tinamou (Tinamidae) and jaçana (Jacanidae) eggshells. *Ibis* **121**, 469–474.

BOARD, R. G. & PERROTT, H. R. (1981). The fine structure of incubated eggs of the Helmeted guinea fowl (*Numidia meleagris*). *Journal of Zoology, London*. (In press).

BOARD, R. G. & SCOTT, V. D. (1980). Porosity of avian eggshells *American Zoologist* **20**, 339–349.

BOARD, R. G. & TULLETT, S. G. (1975). The pore arrangement in the emu (*Dromaius novaehollandiae*) eggshell as shown by plastic models. *Journal of Microscopy* **163**, 281–284.

BOARD, R. G., LOSEBY, S. & MILES, V. R. (1979). A note on microbial growth on hen eggshells. *British Poultry Science* **20**, 413–420.

BOARD, R. G., TULLETT, S. G. & PERROTT, H. R. (1977). An arbitrary classification of the pore system in avian eggshells. *Journal of Zoology, London* **182**, 251–265.

BOARD, R. G., PERROTT, H. R., LOVE, G. & SCOTT, V. D. (1981). The fine structure of the eggshells of Great Crested Grebe (*Podiceps cristalus*). Submitted to *Calcified Tissue International*.

BOND, G. M. (1980). *Avian eggshells and the hatching process*. Ph. D. Thesis, University of Bath.

CALDER, W. A. (1978). The kiwi: a case of compensating divergences from allometric predictions. In *Respiratory Function in Birds, Adult and Embryonic* (ed. J. Piiper), pp. 239–242. Springer-Verlag: Berlin.

CAREY, C. (1979). Increase in conductance to water vapour during incubation in eggs of two avian species. *Journal of Experimental Zoology* **209**, 181–186.

CAREY, C. (1980). Adaptation of the avian egg to high altitude. *American Zoologist* **20**, 449–459.

CARTER, T. C. (1979). The hen's egg: evidence on the mechanism relating shell strength to loading rate. *British Poultry Science* **20**, 175–183.

CRACRAFT, J. (1973). Continental drift, paleoclimatology, and the evolution and biogeography of birds. *Journal of Zoology, London* **169**, 455–545.

DRENT, R. H. (1970). Functional aspects of incubation in the herring gull. *Behaviour Supplement* **17**, 1–132.

ERBEN, H. K. (1970). Ultrastrukturen und Mineralization rezentrer und fossiler Eischalen bei Vögeln und Reptilien. *Biomineralization Forschungsberichte* **1**, 1–66.

FROMM, D. (1959). A rapid method for the determination of egg shell permeability. *Poultry Science* **38**, 171–173.

FUJII, S. (1974). Further morphological studies on the formation and structure of hen's eggshell by scanning electron microscopy. *Journal of the Faculty of Fish and Animal Husbandry, Hiroshima University* **13**, 29–56.

HANKA, L. A., PACKARD, G. C., SOTHERLAND, P. R., TAIGEN, T. L., BOARDMAN, T. J. & PACKARD, M. J. (1979). Ontogenetic changes in water-vapour conductance of eggs of yellow-headed blackbirds (*Xanthocephalus xanthocephalus*). *Journal of Experimental Zoology* **210**, 183–188.

HOWEY, P. W., BOARD, R. G. & KEAR, J. (1977). A pulse-position modulated multichannel radio telemetry system for the study of the avian microclimate. *Biotelemetry* **4**, 169–180.

HOYT, D. F., BOARD, R. G., RAHN, H. & PAGANELLI, C. V. (1979). The eggs of the anatidae: conductance, pore structure and metabolism. *Physiological Zoology* **52**, 438–450.

LACK, D. (1968). *Ecological adaptations for breeding in birds*. Chapman and Hall: London.

LOMHOLT, J. P. (1976). Relationship of weight loss to ambient humidity of birds' eggs during incubation. *Journal of Comparative Physiology* **105**, 189–196.

MONGIN, P. & CARTER, N. W. (1977). Studies on the avian shell gland during egg formation: aqueous and electrolytic composition of the mucosa. *British Poultry Science* **18**, 339–351.

NEEDHAM, J. (1931). *Chemical embryology*. 3 vols. Cambridge University Press, Cambridge.

PACKARD, S. C., SOTHERLAND, P. R. & PACKARD, M. J. (1977). Adaptive reduction in permeability of avian eggshells to water vapour at high altitudes. *Nature, London* **266**, 255–256.

PAGANELLI, C. V. (1980). The physics of gas exchange across the avian eggshell. *American Zoologist* **20**, 329–338.

PAGANELLI, C. V., ACKERMAN, R. A. & RAHN, H. (1978). The avian egg: *in vivo* conductances to oxygen, carbon dioxide, and water vapour in late development. In *Respiratory Function in Birds, Adult and Embryonic* (ed. J. Piiper), pp. 212–218. Springer-Verlag: Berlin.

PERROTT, H. R., SCOTT, V. D. & BOARD, R. G. (1980). Crystal orientation in the shell of the domestic fowl: an electron diffraction study. *Calcified Tissue International* **33**, 119–124.

PRAGER, E. M., WILSON, A. C., OSUGA, D. T. & FEENEY, R. E. (1976). Evolution of flightless land birds on southern continents: transferrin comparison shows monoplyletic origin of ratites. *Journal of Molecular Evolution* **8**, 283–294.

RAHN, H. & AR, A. (1980). Gas exchange of the avian egg: time, stucture and function. *American Zoologist* **20**, 477–484.

RAHN, H., ACKERMAN, R. A. & PAGANELLI, C. V. (1977). Humidity in the avian nest and egg water loss during incubation. *Physiological Zoology* **50**, 269–283.

RAHN, H. CAREY, C., BALMAS, K., BHATIA, B. & PAGANELLI, C. V. (1977). Reduction of pore area of the avian eggshell as an adaptation to altitude. *Proceedings of the National Academy of Science, USA* **74**, 3075–3078.

RAHN, H., PAGANELLI, C. V. & AR, A. (1974). The avian egg: air–cell gas tension, metabolism and incubation time. *Respiration Physiology* **22**, 297–309.

RATCLIFFE, D. A. (1970). Changes attributable to pesticides in egg breakage frequency and eggshell thickness in some British birds. *Journal of Applied Ecology* **7**, 67–115.

SIMKISS, K. (1980). Eggshell porosity and the metabolism of the chick embyro. *Journal of Zoology, London* **192**, 1–8.

SOTHERLAND, P. R., PACKARD, G. C., TAIGEN, T. L. & BOARDMAN, T. J. (1980). An altitudinal cline in conductance of cliff swallow (*Petrochelidon pyrrohonota*) eggs to water vapour. *The Auk* **97**, 177–185.

TAIGEN, T. L., PACKARD, G. C., SOTHERLAND, P. R., BOARDMAN, T. J. & PACKARD, M. J. (1980). Water vapour conductance of Blackbilled magpie (*Pica pica*) eggs collected along an altitudinal cline. *Physiological Zoology* **53**, 163–169.

TULLETT, S. G. (1975). Regulation of avian eggshell porosity. *Journal of Zoology, London* **117**, 339–348.

TULLETT, S. G. (1976). *The avian eggshell: a mediating boundary*. Ph.D. Thesis: University of Bath.

TULLETT, S. G. (1978). Pore size versus pore number in avian eggshells. In *Respiratory Function in Birds, Adult and Embryonic* (ed. J. Piiper), pp. 217–226. Springer-Verlag: Berlin.

TULLETT, S. G. & BOARD, R. G. (1977). Determinants of avian eggshell porosity. *Journal of Zoology, London* **183**, 203–211.

TULLETT, S. G., BOARD, R. G., LOVE, G., PERROTT, H. R. & SCOTT, V. D. (1976). Vaterite deposition during eggshell formation in the cormorant, gannet and shag, and in the 'shell-less' eggs of the domestic fowl. *Acta zoologica, Stockholm* **57**, 79–87.

TYLER, C. (1953). Studies on eggshells. II. A method for marking and counting pores. *Journal of the Science of Food and Agriculture* **4**, 266–272.

TYLER, C. (1964). A study of the eggshells of the Anatidae. *Proceedings of the Zoological Society, London* **142**, 547–583.

TYLER, C. (1965). A study of the eggshells of the Sphenisciformes. *Journal of Zoology, London* **147**, 1–19.

TYLER, C. (1966). A study of the eggshells of the Falconiformes. *Journal of Zoology, London* **150**, 413–425.

TYLER, C. (1969*a*). a study of the eggshells of the *Gaviiformes*, *Procelloriformes*, *Podicipitiformes* and *Pelecaniformes*. *Journal of Zoology, London* **158**, 395–412.

TYLER, C. (1969*b*). Avian eggshells: their structure and characteristics. *International Review of General and Experimental Zoology* **4**, 81–130.

TYLER, C. (1969 *c*). The snapping strength of the eggshells of various orders of birds. *Journal of Zoology, London* **159**, 65–77.

TYLER, C. & FOWLER, S. (1978). The distribution of organic cores, cones, cone junctions and pores in the eggshells of wild birds. *Journal of Zoology, London* **186**, 1–14.

TYLER, C. & FOWLER, S. (1979). The size, shape and orientation of pore grooves in the eggshells of *Rhea* sp. *Journal of Zoology, London* **187**, 283–290.

TYLER, C. & SIMKISS, K. (1959). A study of the eggshells of ratite birds. *Proceedings of the Zoological Society London* **133**, 201–243.

VLECK, C. M. & KENAGY, G. J. (1980). Embryonic metabolism of the Fork-tailed storm petrel: physiological patterns during prolonged and interrupted incubation. *Physiological Zoology* **53**, 32–42.

VLECK, D., VLECK, C. M. & HOYT, D. F. (1980). Metabolism of avian embryos: ontogeny of oxygen consumption in the Rhea and Emu. *Physiological Zoology* **53**, 125–135.

WILBUR, K. M. & SIMKISS, K. (1968). Calcified shells. *Comprehensive Biochemistry* 229–295.

WHITE, F. N. & KINNEY, J. L. (1974). Avian incubation. *Science N.Y.* **186**, 107–115.

WHITTOW, G. C. (1980). Physiological and ecological correlates of prolonged incubation of sea birds. *American Zoologist* **20**, 427–436.

WYBURN, G. M., JOHNSTON, H. S., DRAPER, M. H. & DAVIDSON, M. F. (1973). The ultrastructure of the shell-forming region of the oviduct and the development of the shell of *Gallus domesticus*. *Quarterly Journal of Experimental Physiology* **58**, 143–151.

Summary

Field observations in Britain, Iceland and Japan showed that Whooper Swans *Cygnus c. cygnus* at the eastern end of their range have more yellow on their bills than do those at the western extreme. While the bill patterns of Bewick's *C. columbianus bewickii* and Whistling Swans *C. columbianus columbianus* are most similar where their ranges are closest, those of the Whooper and Trumpeter Swans *C. c. buccinator* are then most different. The latter pair may have diverged earlier or have remained more consistently isolated.

References

Anon. 1978. The Birds are Coming Back. *Japan Pictorial* 1: 8–13.
Evans, M. E. 1977. Recognizing individual Bewick's Swans by bill pattern. *Wildfowl* 28: 153–8.
Evans, M. E. & Sladen, W. J. L. 1980. A comparative study of the bill markings of Whistling and Bewick's Swans and out-of-range occurrences of the two taxa. *Auk* 97: 697–703.
Scott, D. K. 1978. Identification of Individual Bewick's Swans by bill pattern. In B. Stonehouse (ed.) *Animal Marking*. London: Macmillan.
Scott, D. K. 1981. Geographical variation in the bill patterns of Bewick's Swans (*Cygnus columbianus bewickii*). *Wildfowl* 32: 123–8
Scott, P. 1966. The Bewick's Swans at Slimbridge. *Wildfowl Trust Ann. Rep.* 17: 20–26.

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A 'yellowneb' Whooper Swan *Cygnus cygnus* showing a black brow. (Dr L. W. S. Eyears)

The microstructure of avian eggshells, adaptive significance and practical implications in aviculture

R. G. BOARD

Introduction

In a discussion of 'problems concerned with eggs', Lack (1968) concluded that the following features of eggshells were probably adaptations for waterproofing: the chalky films on eggshells of grebes (Podicipedidae) and cormorants (Phalacrocorciidae); the powdery cover on those of flamingoes (Phoenicopteridae); the greasy surface of the duck's (Anatidae) egg, and the polished one of the lily-trotter (Jacanidae). The reasons for waterproofing were not discussed. The application of electron optics has shown that all but one of these eggshells share a common morphological feature, a layer of spheres on their outer surfaces (Board 1980; Board *et al.* 1977). The exception, the Lily-trotter *Micropara capensis*, has a dense crystalline outer border to the calcitic shell and the bell-shaped outer orifice of the pore canal is roughly plugged with amorphous material rich in sulphur (Board & Perrott 1979*a*). Although spheres are common to the other groups, chemical differences do occur. The spheres on grebes' eggshells contain amorphous calcium phosphate (Board *et al.* 1981) whereas those on the cormorant's shell contain an unusual form of calcium carbonate, vaterite (Tullett *et al.* 1976; Board & Perrott 1979*b*). It will be noted below that flamingo eggshells are covered with spheres containing amorphous calcium phosphate and a material rich in sulphur.

This communication has three objectives: (i) to discuss the problems faced by eggs laid in wet or muddy nests, (ii) to encourage field studies of the interplay between the microstructure of eggshells, the nest environment and bird behaviour, and (iii) to alert aviculturists to the possible danger in adopting the wrong methods for cleaning and disinfecting eggshells.

Problems faced by eggs

Independence of a need for external water can be considered the last major step in the evolution of the cleidoic eggs of birds (Needham 1931). Although such an egg i self-contained in respect of available wate and nutrients for embryo development, it shell must be porous in order that th embryo can exchange respiratory gase with the nest environment. As the oxyge molecule is larger than that of water, th pores allow diffusion of water vapour an thereby depletion of the reservoir of wate present when laid. Drent's (1975) review c field observations indicates that 16%, o average, of the reservoir is lost durin incubation. The classic studies of Rahn an his collaborators (e.g. Ar & Rahn 1978 have shown that this loss is the outcome c an interplay between shell conductanc (porosity), egg mass, nest humidity, in cubation period and barometric pressure a the nest site. Indeed the weight of evidenc can easily lead to the conclusion that avia eggshells are precisely adapted for wate conservation, and that an embryo's re quirements for oxygen are of secondar importance. The emphasis given to wate conservation has been challenged by Sim kiss (1980) who successfully hatched chick of the domestic hen even though the allan tois had been drained about two-thirds th way through incubation.

The cross-sectional area of an eggshe pore canal is never more than a few squar microns and the majority of pores mus remain open throughout incubation if th embryo's demands for oxygen are to b satisfied. A problem of equal importanc to that of water conservation, therefore, i how the pore canals do not become block ed by mud, preening oils, nest debris or th dust arising from attrition between eggs. I this context, waterproofing would be bu one of the prerequisites of a shell. Indee there appears to be no record of waterlog ging of shells leading to the asphyxiation c embryos, but the experiences of the pou try industry leave no doubt that the flooc ing of a few pores with contaminated wate is the first step in the process leading to th addling of eggs (Board 1980; Board & Halls 1973). Moreover glycoproteins in th cuticle on hens' eggshells are colonized an digested by bacteria if the storage cond tions are very humid (Board *et al.* 1979

Thus it would seem reasonable to assume that adaptations of eggshells will be influenced by the nest environment.

The nest of a flamingo, a mud platform alongside water rich in minerals, would offer a harsh environment to eggshells lacking appropriate adaptations. For example, if the developing embryo were contained in a calcitic shell in which the open orifice of the pore canal was flush with the shell's surface, as is in the pigeon *Columbia livia* (Board 1974), then blockage of the pores with mud, debris or the crystals remaining after evaporation of liquid brought to the nest by the parents would be a distinct possibility. The following discussion of the microstructure of flamingo eggshells supports the contention that they are adapted to counter these inimical features.

Flamingo eggshells

Examination of the eggshells of all extant species of flamingoes has shown that they share a common structure. The outer surface of the eggshell and the outer orifice of the pore canal are covered (Figure 1) with a thick layer of spheres (Figure 2); the transition between the calcitic shell and spheres is abrupt (Figure 3). Electron probe analysis has shown that the spheres are rich in sulphur and phosphorus (Figure 4), infra-red analysis that calcium phosphate is a component of the spheres, and X-ray diffraction studies that this phosphate is non crystalline.

If the structure shown in Figure 1 is considered as a diffusion pathway for res-

Figure 2. Details of the spheres occurring on the outer surface of the shell of Greater Flamingo. Bar marker, 2·5 μm.

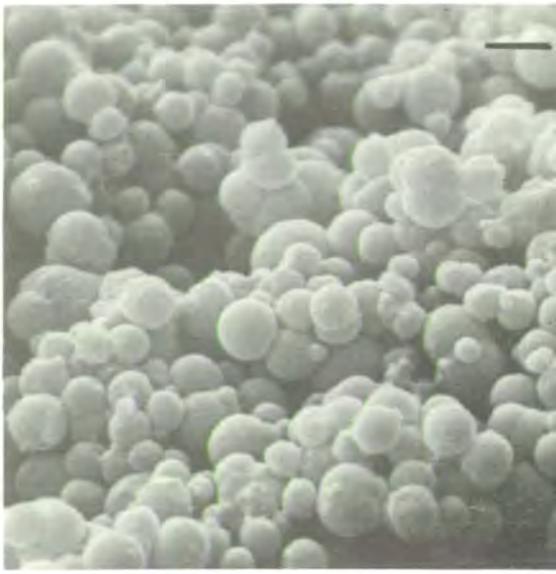


Figure 1. The radial face of a piece of snapped shell of Greater Flamingo as seen with a scanning electron microscope. Bar marker, 1000 μm. S, layer of spheres; P, pore canal; C, cone layer, and SM, shell membranes. The numbers refer to probe sampling sites, see Figure 4.

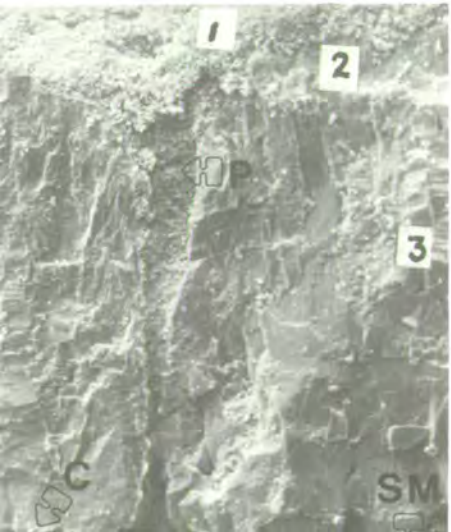
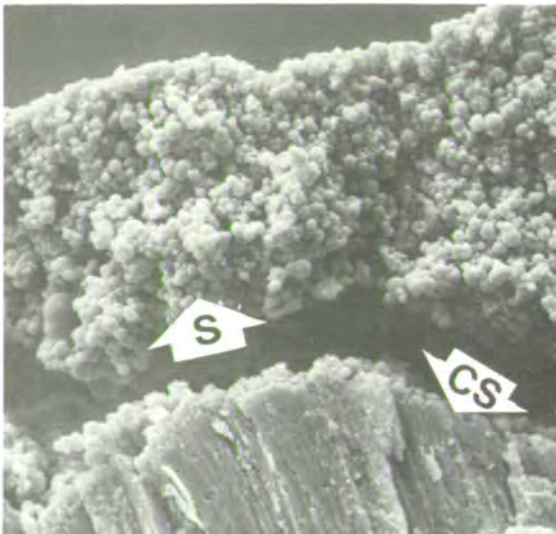


Figure 3. A scanning electromicrograph showing the abrupt change from the calcitic shell (CS) to the outer layer of spheres (S) on the eggshell of Greater Flamingo. Bar marker, 1 μm.



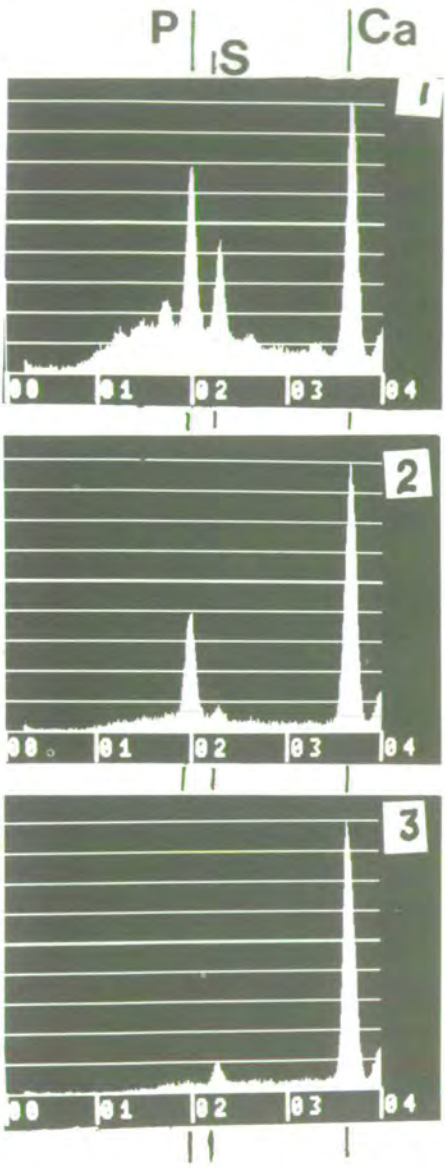


Figure 4. Elemental analysis of the eggshell of Greater Flamingo by electron probe analysis. Point analyses were done at the sites noted in Figure 1. The spheres contained relatively large amounts of calcium, sulphur and phosphorus (1). The outer edge of the calcitic shell contained calcium, phosphorus and a trace of sulphur (2) whereas the central region (in radial plane) of the shell contained no demonstrable amount of phosphorus, a small amount of sulphur and a large amount of calcium (3).

piratory gases and water vapour (Figure 5), then a series of resistances to the inward flux of oxygen can be identified: R_1, the layer of spheres on the shell surface; R_2, the pore canal, and R_3, the shell membranes. Judging from the studies of the role of glycoprotein spheres on the eggshells of domestic hens (Board 1980; Board & Halls 1973), R_1 can be tentatively identified as the resistance that prevents flooding of the pore canals. Such an interpretation would be in accord with that of Lack (1968). This resistance would also prevent the outer orifices of the pore canals from becoming blocked with extraneous materials. As the deposition of mud, debris, crystals etc could be expected to increase the resistance that R_1 offered to the flux of respiratory gases, the question arises: how is this resistance prevented from attaining a value that would impede the embryo's exchange

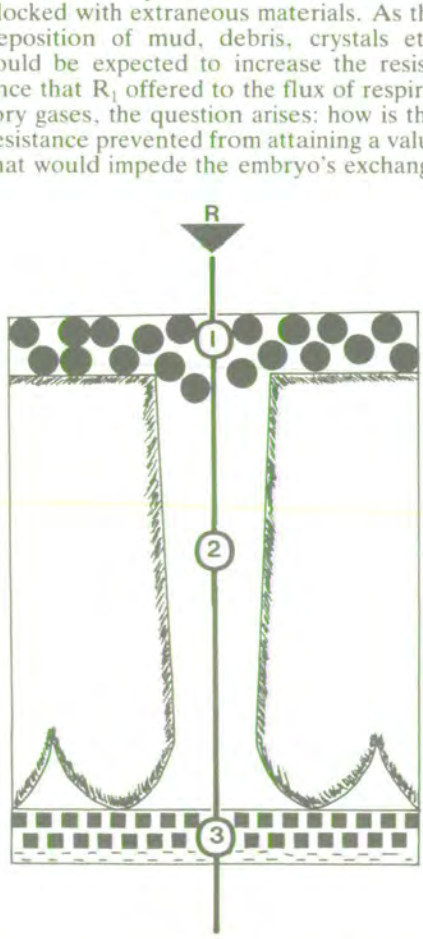


Figure 5. The eggshell of Greater Flamingo considered as a resistance network. Three major resistances, acting in series, are recognized: R_1, the layer of spheres on the outer surface of the shell; R_2, the pore canal, and R_3, the shell membranes.

of respiratory gases with the nest atmosphere? Several possible answers suggest themselves. For example, the deposition of debris on the surface of R_1 might increase the resistance locally such that the relatively direct diffusion pathway depicted in Figure 5 could not function; the pore canals would exchange with the void spaces in the sphere layer and these would exchange across the entire outer surface of the powdery layer. Alternatively the fissures present in the outer surface of this layer may be kept open as a consequence of egg turning, a possibility suggested by studies of the incubated eggs of Grebes (Board *et al.* 1981). A third possibility would be that through turning, the outer surface of the powdery layer is progressively worn down so that R_1 never offers a serious impediment to the flow of respiratory gases yet its thickness never diminishes to such an extent that the waterproofing of the eggshell is impaired.

Field studies

There would thus appear to be *a priori* reasons for assuming that eggshells are adapted to the nest environment and also to features resulting from the parents' own adaptations. Preening oils, for example, could presumably cause resistance R_1 (Figure 5) to increase progressively during incubation. Moreover attrition between eggs during turning can modify the shell's surface. Thus the cuticle on the shells of the Helmeted Guinea-fowl *Numidia meleagris* is worn away during incubation but the counter-sunk orifice of the pore canals protects the plug of cuticular material (Board & Perrott 1981). As this plug becomes stained during brooding by the parent, it would appear to be operating as a filter such that extraneous materials do not block the pore canals or contaminate the shell membranes. These observations suggest that there is a need to shift the emphasis in field studies concerned with the breeding biology of birds. To date much attention has been given to those features of bird behaviour that ensures heating, turning and protection of the eggs (White & Kinney 1974; Drent 1975; Howey *et al.* 1977). The present discussion suggests that behaviour alone cannot assure the well-being of the developing embryo and that shell adaptations compensate for those factors that the parents cannot control or, on occasions, may even accentuate.

One possible approach in field studies would be to modify eggshells—i.e. remove the layer of spheres on the eggshells of waterfowl—and monitor weight loss with them. Changes in the fine structure of the shell's surface during incubation would be another approach and the non-destructive technique introduced by Board (1981*a*) could make a useful contribution to such studies. The swapping of eggs could be another approach but probably the most useful method, as suggested by Board (1981*b*), would be the use of dummy eggs to measure changes in the conductance and the content of extraneous material of the 'shell'.

Practical implications

It would seem reasonable to assume that those practices adopted by the poultry industry would be appropriate should there be a need to wash eggs. Wash water should be maintained at a temperature greater than that of the egg (Board 1980). The presence of phosphates in the covers on the eggshells of flamingoes would presumably inactivate certain of the quarternary ammonium compounds that are used as disinfectants. Thus there would appear to be a need for studies of disinfectants so that ones appropriate for the treatment of waterfowl eggs can be identified.

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Summary

Eggs laid in 'wet places' need to be adapted so that the pore canals in the calcareous shell are not flooded with water or occluded with mud, nest debris, preening oils or salts. The shell of the Greater Flamingo *Phoenicopterus ruber roseus* was taken as an example. Its surface layer of spheres rich in calcium, phosphorus and sulphur is probably the adaptation that fits the egg to the nest environment. Field studies could establish the extent of the contribution of the outer layer of spheres to the well-being of the embryo.

References

Ar. A. & Rahn, H. 1978. Interdependence of gas conductance, incubation length and weight of avian egg. Pp. 227–38 *in* J. Piiper (ed.) *Respiratory function in birds, adult and embryonic*. Berlin: Springer.

Board, R. G. 1974. Microstructure, water resistance and water repellency of the pigeon eggshell. *Br. Poult. Sci.* 15: 415–9.

Board, R. G. 1980. The avian eggshell: a resistance network. *J. appl. Bact.* 48: 303–13.

Board, R. G. 1981a. A non-destructive method for studying the outer surface of avian eggshells. *Br. Poult. Sci.* (In press).

Board, R. G. 1981b. Problems concerned with avian eggs. Submitted to *Biol. Rev.*

Board, R. G. & Halls, N. A. 1973. The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poult. Sci.* 14: 69–97.

Board, R. G. & Perrott, H. R. 1979a. The plugged pores of tinamou (Tinamidae) and Jaçana (Jananidae) eggshells. *Ibis* 121: 469–74.

Board, R. G. & Perrott, H. R. 1979b. Vaterite, a constituent of the eggshells of the non-parasitic Cuckoos, *Guira guira* and *Crotophagi ani*. *Calc. Tissue Intl.* 29: 63–69.

Board, R. G. & Perrott, H. R. 1981. The fine structure of incubated eggshells of the Helmeted guinea fowl (*Numidia meleagris*) submitted to *J. Zool., Lond.*

Board, R. G., Loseby, S. and Miles, V. R. 1979. A note on microbial growth on the avian eggshell. *Br. Poult. Sci.* 20: 413–20.

Board, R. G., Tullett, S. G. and Perrott, H. R. 1977. An arbitrary classification of the pore systems in avian eggshells. *J. Zool., Lond.* 182: 251–65.

Board, R. G., Perrott, H. R., Love, G. and Scott, V. D. 1981. The phosphate rich cover of the eggshells of the Great Crested Grebe, *Podiceps cristatus*, and related species. submitted to *Calcif. Tiss. Int.*

Drent, R. H. 1975. Incubation. Pp. 333–420 *in* D. S. Farner and J. R. King (ed.) *Avian Biology* Vol. 5. New York: Academic Press.

Howey, P. W., Board, R. G. & Kear, J. 1977. A pulse position modulated multi-channel radio telementry system for the study of the avian nest microclimate. *Biotelemetry* 5: 169–80.

Lack, R. 1968. *Ecological adaptations for breeding in birds*. London: Chapman and Hall.

Needham, J. 1931. *Chemical embryology*. 3 Vols. Cambridge: University Press.

Simkiss, K. 1980. Eggshell porosity and water metabolism of the chick embryo. *J. Zool., Lond.* 192: 1–8.

Tullett, S. G., Board, R. G., Love, G., Perrott, H. R. & Scott, V. D. 1976. Vaterite deposition during eggshell formation in the cormorant, gannet and shag, and in some "shell-less" eggs of the domestic fowl. *Acta Zool., Stockh.* 57: 79–87.

White, F. N. & Kinney, J. L. 1974. Avian incubation. *Science, N.Y.* 186: 107–15.

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CORRELATION OF HATCHING TECHNIQUES IN SOME AVIAN SPECIES WITH THE MECHANICAL PROPERTIES OF THEIR EGGS

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Eggs from the domestic hen, domestic duck, Japanese quail and feral pigeon were selected for study, since earlier work (Oppenheim, 1972) indicated that they exhibited differences in hatching characteristics.

Unincubated eggs were tested in compression between flat plates. Pipping and hatching simulations, in which loading was on the inner surface of the shell, were performed on half-shells of incubated eggs mounted on annuli of resin in an Instron compressive test rig. With hen and duck eggs, the onset of fracture is followed by a marked and rapid decrease in load (Fig. 1*a*) corresponding to the development of a crack which, with brief arrest periods, spreads across the shell. In contrast, quail and pigeon eggs show no sharp peak in the load–displacement curve (Fig. 1*b*) but continue to support substantial loads as cracks develop. Hen eggs, which have relatively low energy requirements for crack propagation, produce comparatively few large acoustic emissions during fracture (Fig. 2*a*). Quail eggs, however, give greater numbers of large acoustic emissions (Fig. 2*b*), associated with many short, energy-consuming steps in crack growth.

We can thus make a clear distinction between eggs of different species based upon their mechanical characteristics. The rapid and catastrophic propagation of a single crack together with the accompanying loss of strength of the whole structure once fracture has been initiated, as observed in the hen and duck eggs, are typical of brittle failure. With the quail and pigeon eggs, however, cracks formed at the onset of fracture remain short and can be extended only by further application of fairly substantial loads; crack propagation occurs in short bursts and more cracks are likely to be generated. Such behaviour is characteristic of a tough, more flexible material. It should be noted that the above results refer to eggshells with membranes present. Removal of the shell

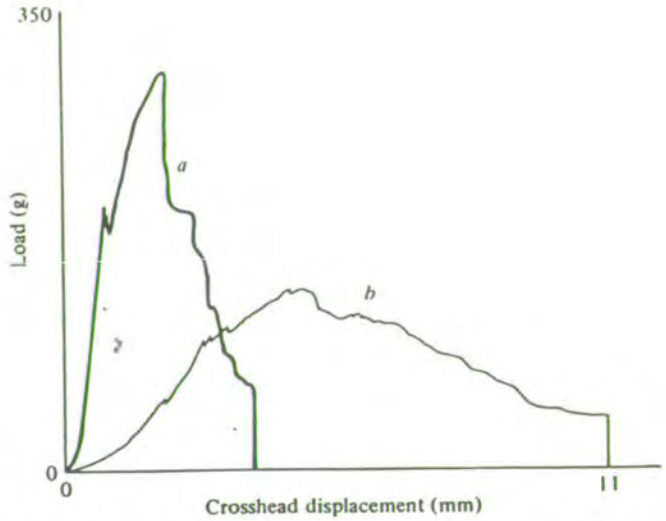

Fig. 1. Hatching simulation with egg halves on an Instron tensometer: *a*, hen; *b*, quail (see text for details).

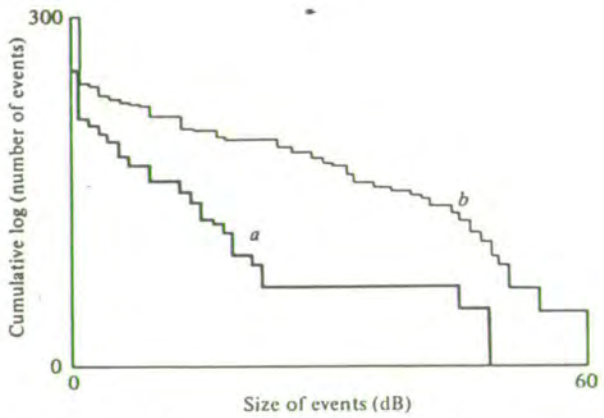

Fig. 2. Acoustic-emission data from compression of a whole, blown, eggs. *a*, hen; *b*, quail.

membranes prior to pipping or hatching simulations lessens but does not eliminate the distinction between brittle and tough behaviour.

We have studied the hatching techniques of a range of species in the laboratory, at bird-breeding establishments and in the field, and have looked at films of hatching. It appears that two main types of hatching techniques are exhibited. For example, hen and duck neonates, after pipping when the initial fracture occurs, may chip away at the shell to

produce a small hole. Further cracking is induced until the damage extends for approximately two thirds of the circumference of the shell, and then the cap is pushed off to allow the chick to emerge. Quail and pigeon neonates, however, chip virtually the whole way round the egg, perforating shell and membranes at quite small intervals, before pushing off the cap; the quail may take more than one revolution to achieve adequate perforation (Oppenheim, 1972).

These two broad categories of hatching technique correlate with the classification of the four species based upon the mechanical test and acoustic emission data from their eggs. With hen and duck eggs a crack, once produced, can be propagated fairly easily and the hatching technique of the chick consists of making relatively few initial cracks before pushing off the cap. By contrast the strength of the tougher eggs of quail and pigeon does not decrease greatly after the initial damage and, consequently, chicks have to assist crack propagation by making an extensive series of cracks or holes around the circumference before they can push off the cap.

These results form the basis for a wider spectrum of behaviour. At one extreme would lie species such as the quail, pigeon and little bittern (Zwergrohrdommel, 1962), whose eggs display toughness, while at the other would lie species such as the rhea and ostrich whose eggs are very hard and brittle and whose chicks chip, at most, a quarter of the way round the egg before pushing away a cap (Bruning, 1974; BBC, 1977).

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References

BBC, (1977). *Namib – strange creatures of the skeleton coast.* 'World About Us' series, film no. 15222.

Bruning, D. F. (1975). Social structure and reproductive behaviour in the greater rhea. *The Living Bird, Thirteenth Annual, 1974*, pp. 251–94.

Oppenheim, R. W. (1972). Prehatching and hatching behaviour in birds: a comparative study of altricial and precocial species. *Animal Behaviour*, **20**, 644–55.

Zwergrohrdommel, X. Y. (1962). *Ixobrychus minutus (Ardeïdae) – Schlüpfen, erste Lebensstunde* [Hatching, first hour of life. Little bittern]. From *Encyclopeadia Cinematographica*, film no. E275. Göttingen: Institut für der Wissenschaftlichen Film.

LITERATURE CITED

AR, A., C. V. PAGANELLI, R. B. REEVES, D. G. GREENE, and H. RAHN. 1974. The avian egg: water vapor conductance, shell thickness, and functional pore area. Condor **76**:153–158.

AR, A., and H. RAHN. 1978. Interdependence of gas conductance, incubation length and weight of the avian egg. Pages 227–236 *in* J. PIIPER, ed. Respiratory function in birds, aduit and embryonic. Springer, Berlin.

BOARD, R. G., S. G. TULLETT, and H. R. PERROTT. 1977. An arbitrary classification of the pore systems in avian eggshells. J. Zool. **182**:251–265.

DRENT, R. 1970. Functional aspects of incubation in the Herring Gull. Behaviour **17**(suppl.):1–132.

———. 1975. Incubation. Pages 333–420 *in* D. S. FARNER and J. R. KING, eds. Avian biology. Vol. 5. Academic Press, New York.

HOYT, D. F. 1976. The effect of shape on the surface-volume relationship of birds' eggs. Condor **78**:343–349.

———. 1979. Practical methods of estimating the volume and fresh weight of birds eggs. Auk **96**: 73–77.

HOYT, D. F., D. VLECK, and C. M. VLECK. 1978. Metabolism of avian embryos: ontogeny and temperature effects in the ostrich. Condor **80**: 265–271.

KUTCHAI, H., and J. B. STEEN. 1971. Permeability of the shell and shell membranes of hens' eggs during development. Respiration Physiol. **11**: 265–278.

LOMHOLT, J. P. 1976*a*. The development of the oxygen permeability of the avian eggshell and its membranes during incubation. J. Exp. Zool. **198**:177–184.

———. 1976*b*. The relationship of weight-loss to ambient humidity of birds' eggs during incubation. J. Comp. Physiol. **105**:189–196.

PAGANELLI, C. V., R. A. ACKERMAN, and H. RAHN. 1978. The avian egg: *in vivo* conductances to O_2, CO_2 and water vapor in late development. Pages 212–218 *in* J. PIIPER, ed. Respiratory function in birds, adult and embryonic. Springer, Berlin.

PAGANELLI, C. V., A. AR, H. RAHN, and O. D. WANGENSTEEN. 1975. Diffusion in the gas phase: the effects of ambient pressure and gas composition. Respiration Physiol. **25**:247–258.

RAHN, H., and A. AR. 1974. The avian egg: incubation time and water loss. Condor **76**:147–152.

RAHN, H., C. V. PAGANELLI, and A. AR. 1974. The avian egg: air-cell gas tension, metabolism and incubation time. Respiration Physiol. **22**:297–309.

RAHN, H., C. V. PAGANELLI, I. C. T. NISBET, and G. C. WHITTOW. 1976. Regulation of incubation water loss in eggs of seven species of terns. Physiol. Zool. **49**:245–259.

ROMANOFF, A. L., and A. J. ROMANOFF. 1949. The avian egg. Wiley, New York.

SCHOLANDER, P. F. 1950. Volumetric plastic micro respirometer. Rev. Sci. Instruments **21**:378–380.

SCHÖNWETTER, M. 1960–1961. Pages 112–134 *in* W. MEISE, ed. Handbuch der Oologie. Akademie Verlag, Berlin.

TULLETT, S. G. 1975. Regulation of avian eggshell porosity. J. Zool. **177**:339–348.

———. 1976. The avian eggshell: a mediating boundary. Ph.D. diss. Bath University, Bath, England.

TULLETT, S. G., and R. G. BOARD. 1976. Oxygen flux across the integument of the avian egg during incubation. Brit. Poultry Sci. **17**:441–450.

———. 1977. Determinants of avian eggshell porosity. J. Zool. **183**:203–211.

TYLER, C. 1953. Studies on egg shells. II. A method for marking and counting pores. J. Sci. Food Agr. **4**:266–272.

———. 1964. A study of the egg shells of the Anatidae. Proc. Zool. Soc. London **142**:547–583.

VLECK, D., C. M. VLECK, and D. F. HOYT. 1979. Metabolism of avian embryos: ontogeny of oxygen consumption in the Emu and Rhea. Physiol. Zool. **52**:363–377.

WANGENSTEEN, O. D., and H. RAHN. 1970/1971. Respiratory gas exchange by the avian embryo. Respiration Physiol. **11**:31–45.

TABLE 4

COMPARISON OF OBSERVED AND CALCULATED AIR-CELL GAS TENSIONS

Species	No.	$P_{A_{O_2}}$		$P_{A_{CO_2}}$	
		Calculated	Observed	Calculated	Observed
Anas clypeata[a]	3	95	92	46	53
Dendrocygna autumnalis	2	115	122	29	26
Aytha fuligula[a]	4	89	108	54	41
Anser canagicus	2	107	109	38	42

NOTE.—No. = sample size for observed value; calculated values are from table 3.
[a] G_{H_2O} calculated from $5.15 \cdot W/I$ (see table 1).

gests that in other species which have unusually high water-vapor conductances (e.g., Grebe and Coot [Lomholt 1976*b*]) the air-cell gas tensions may differ from the average values.

Vleck et al. (1979) have reported that, just prior to pipping, the partial pressure of oxygen in the air cell ($P_{A_{O_2}}$) is a function of egg weight. If we exclude the two species of Whistling Ducks, we obtain a similar relationship for the anatidae ($P_{A_{O_2}}$ has units of torr):

$$P_{A_{O_2}} = 73 \cdot W^{0.078}, \qquad (9)$$

no. = 9, $r^2 = .75$, $P < .05$. In spite of the general correspondence between observed and calculated air-cell gas tensions (table 4), we consider equation (9) to be a prediction rather than an observation. This is because the values used in deriving the relationship were calculated from conductance and the rate of oxygen consumption.

A "TYPICAL" ANATID EGG

One way to summarize our results is to provide a description (table 5) of the morphological and functional characteristics of a "typical" anatid egg. We arbitrarily selected 80 g as the weight of this typical egg because this is about the mean weight of the eggs in our study.

APPENDIX

The calculation of G_{O_2} (at 38 C) from G_{H_2O} (measured at 25 C) involves three different conversions:

1. To convert G_{H_2O} into units of volume, multiply by the ratio of the molar volume of an ideal gas to the molecular weight of water (22.414/18.02 = 1.244). Thus 1.244 G_{H_2O} (mg day^{-1} torr^{-1}) = G_{H_2O} (cm^3 STP day^{-1} torr^{-1}).
2. To correct G for the temperature effect on the diffusion coefficient, multiply by the square root of the ratio of the absolute temperatures $[(311/298)^{1/2} = 1.022]$. Thus $G_{H_2O}{}^{38°} = 1.002\ G_{H_2O}{}^{25°}$.
3. To correct G for the differences between the diffusion coefficients of water vapor and O_2, multiply by ratio of the diffusion coefficients at 38 C (.23/.27 = .85). Thus $G_{O_2}{}^{38°} = .85\ G_{H_2O}{}^{38°}$.

Combining all corrections yields $G_{O_2}{}^{38°} = 1.08\ G_{H_2O}{}^{25°}$.

TABLE 5

THE MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF A "TYPICAL" ANATID EGG

Morphological Characteristics	
Whole egg:	
Weight	80 g
Density	1.08 g·cm^{-3}
Volume	74 cm^3
Surface area	87 cm^2
Pores:	
No. pores	9,475
Area of pore	224 μm^2
Total pore area (A_p)	.02 cm^2
A_p as % of surface area	.024%
Radius of pore	8.4 μm
Length of pore	450 μm
Functional Characteristics	
Incubation period	28 days
Water-vapor conductance	14.8 mg·day^{-1}·torr^{-1}
O_2 conductance	16 cm^3·day^{-1}·torr^{-1}
Pre-IP O_2 consumption	740 cm^3·day^{-1}
Air-cell O_2 tension	103 torr
Air-cell CO_2 tension	41 torr

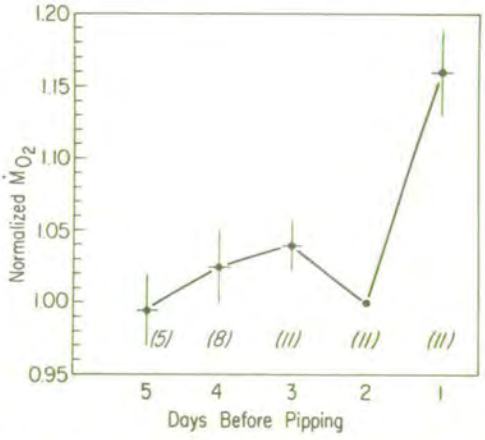

FIG. 5.—A generalized pattern of oxygen consumption near the end of incubation. The species mean rates plotted in figure 4 were normalized by dividing through by the rate observed 2 days before pipping. The horizontal lines indicate the mean normalized rate. The vertical lines indicate 1 SE of the mean. The values observed 3 days and 1 day before pipping are both significantly greater than 1, which, by definition, is the value observed 2 days before pipping. Sample sizes are indicated in parentheses.

AIR-CELL GAS TENSIONS

Air-cell gas tensions ($P_{A_{O_2}}$ and $P_{A_{CO_2}}$) were calculated from water-vapor conductances and the pre-IP rates of oxygen consumption using equations (5) and (6). The mean calculated $P_{A_{O_2}}$ and $P_{A_{CO_2}}$ are 103 ± 11 torr and 41 ± 10 torr, respectively. These values are quite similar to the mean values (104 torr and 37 torr) measured by Rahn et al. (1974).

The air-cell gas tensions measured 2 days before pipping on the eggs of four species are compared with the calculated values for the same species in table 4. The similarity between the observed and calculated values is impressive, particularly in view of the small sample sizes and the fact that the gas tensions, gas conductances, and rates of oxygen consumption were all measured on different groups of eggs. The similarity also supports the validity of equations (5) and (6) and the accuracy of the other calculated gas tensions in table 3.

The air-cell O_2 tensions of the two species of Whistling Ducks (*Dendrocygna bicolor* and *D. autumnalis*) are unusually high, and the CO_2 tensions are low (table 3). The reason for this is that the shell conductances are unusually high (conductance coefficients equal 7.85 and 7.30, respectively [table 1]). This sug-

TABLE 3

PREINTERNAL PIPPING RATES OF OXYGEN CONSUMPTION ($\dot{M}_{O_2}$) AND CALCULATED AIR-CELL GAS TENSIONS ($P_{A_{O_2}}$ and $P_{A_{CO_2}}$)

Species	No.	W (g)	$\dot{M}_{O_2}$ (cm³·Day⁻¹)	SD	G_{H_2O} (mg·Day⁻¹·Torr⁻¹)	$P_{A_{O_2}}$ (Torr)	$P_{A_{CO_2}}$ (Torr)
Calonetta leucophrys (Ringed Teal)	5	29.2	335	7.9	6.1	99	46
Anas cyanoptera (Cinnamon Teal)	6	29.4	376	20.5	6.1[a]	91	51
Anas clypeata (Common Shoveler)	7	39.7	473	52.5	8.5[a]	95	46
Dendrocygna autumnalis (Red-billed Whistling Duck)	4	50.9	410	12.7	11.6	115	29
Dendrocygna bicolor (Fulvous Whistling Duck)	8	51.2	514	41.4	17.1	123	25
Aythya fuligula (Tufted Duck)	5	51.4	669	50.5	10.2[a]	89	54
Netta rufina (Red-crested Pochard)	7	54.3	551	41.9	10.7	100	43
Aythya novaeseelandia (New Zealand Scaup)	4	67.2	626	10.3	10.5	96	49
Anser caerulescens (Lesser Snow Goose)	6	120.0	1,162	52.0	26.9[a]	108	36
Anser canagicus (Emperor Goose)	6	131.8	1,248	63.6	27.4	107	38
Cygnus atratus (Black Swan)	6	255.3	1,450	262.6	36.5[a]	112	33

NOTE.—No. = sample size; W = mean fresh egg weight; SD = standard deviation of $\dot{M}_{O_2}$; G_{H_2O} = water-vapor conductance calculation of air-cell gas tensions in described in "Material and Methods" section.

[a] $G = 5.15 \cdot W/I$.

a generalization. However, 47 of the 64 eggs in our study did exhibit a decline in the rate of oxygen consumption, and the mean value observed 2 days before pipping is significantly lower than those observed 1 and 3 days before pipping ($P < .05$). A similar pattern exists in the O_2 consumption of the eggs of the Ostrich (Hoyt et al. 1978), Rhea, and Emu (Vleck, Vleck, and Hoyt 1979). A decline in the rate of oxygen consumption several days before pipping may be a general phenomenon in the eggs of precocial species and may reflect a decline in growth rate (Hoyt et al. 1978). We will use the pre-IP rate of oxygen consumption for interspecific comparisons because we assume it reflects an analogous phase in the development of the embryos of different species.

The mean pre-IP rates of oxygen consumption for each species are presented in table 3. The allometric relationship between the pre-IP rate of oxygen consumption ($\dot{M}_{O_2}$, cm^3 O_2 STP day^{-1}) and fresh egg weight (W, measured in grams) is:

$$\dot{M}_{O_2} = 31.5 \times W^{0.72}, \qquad (8)$$

$r^2 = .917$. This relationship is quite similar to that reported by Rahn et al. (1974), whose data included three anatid and 13 nonanatid species.

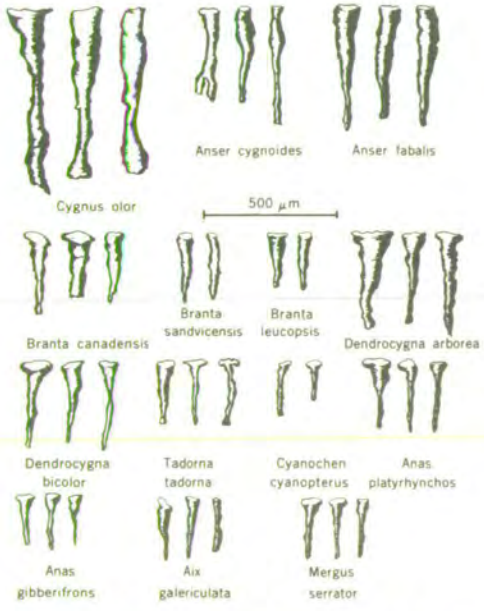

FIG. 2.—Drawings of pore casts from 14 species of anatid eggs. The casts were produced by forcing plastic into the pores and then dissolving the shell. In all cases, the outer, usually flared, end of the pore is up. Redrawn from Tyler (1964).

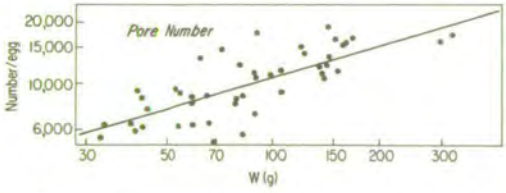

FIG. 3.—Pore number as a function of egg weight. The data are plotted on logarithmic coordinates.

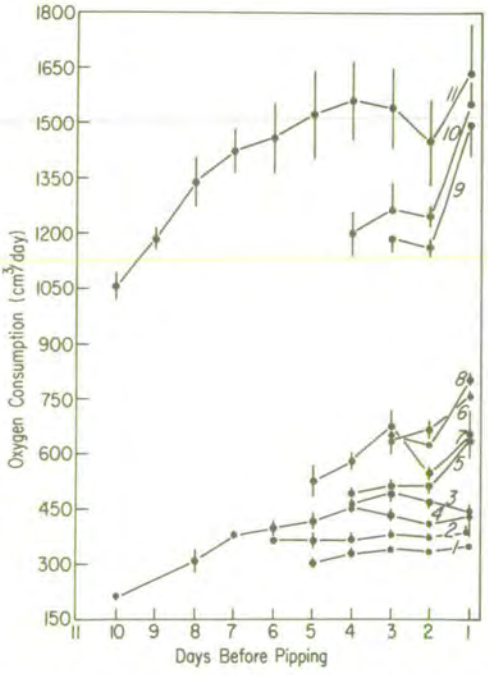

FIG. 4.—The rate of oxygen consumption plotted versus days before pipping. The vertical lines indicate 1 SE of the mean, and the other lines connect consecutive species means. The individual species are indicated by numbers which correspond with the order of species listed in table 3 (i.e., no. 1 = *C. leucophrys*, no. 11 = *C. atratus*).

TABLE 2

COMPARISON OF WATER-VAPOR CONDUCTANCE VALUES OBSERVED (G) AND CALCULATED (G_c) AND THE MORPHOLOGICAL DATA FROM WHICH CONDUCTANCE WAS CALCULATED

Species	Volume (cm^3)	Observed G ($mg \cdot Day^{-1} \cdot Torr^{-1}$)	Morphological Data: N (Pores/Egg)	P_A (μm^2)	L (mm)	Calculated G ($mg \cdot Day^{-1} \cdot Torr^{-1}$)	%E (%)
Aix galericulata	39.8	6.2	6,104	180	.24	10.0	62
Anas gibberifrons	30.8	8.3	5,473	160	.26	7.3	−11
Mergus cucullatus	51.0	11.3	6,182	160	.46	4.7	−59
Dendrocygna autumnalis	40.5	11.5	8,491	90	.34	4.8	−58
Bucephala islandica	63.8	11.9	6,369	490	.39	18.0	50
Dendrocygna arborea	59.6	12.5	8,612	240	.41	11.1	−11
Cyanochen cyanopterus	77.4	14.2	5,563	240	.29	10.3	−27
Tadorna variegata	85.1	14.7	7,049	440	.41	17.2	17
Tadorna tadorna	72.1	14.8	8,386	460	.40	21.8	47
Mergus merganser	63.8	15.0	5,127	440	.32	16.0	7
Anas platyrhynchos fulvigula	51.4	15.9	8,997	350	.28	25.5	60
Branta canadensis minima	92.6	16.7	10,870	80	.36	5.4	−68
Dendrocygna bicolor	50.6	17.9	9,394	140	.34	8.6	−52
Anser erythropus	145.5	20.9	14,964	240	.45	17.7	−15
Anser fabalis	152.2	21.3	16,124	250	.46	19.9	−6
Anser fabalis brachyrynchus	123.9	21.8	10,927	330	.55	14.4	−34
Branta leucopsis	98.2	24.7	11,435	170	.39	11.4	−54
Oxyura leucocephala	90.3	26.0	17,599	230	.37	24.1	−7
Anser anser	145.0	32.7	15,339	170	.49	11.9	−64
Branta sandvicensis	145.4	34.8	11,201	450	.39	29.5	−15
Anser cygnoides	135.7	34.9	18,752	460	.47	41.1	18

NOTE.—Volume = egg volume; N = no. pores/egg; P_A = average area of an individual pore; L = shell thickness; $\%E = 100 \cdot (G_c - G)/G$ (G_c calculated according to eq. [3]); $mm^2 = \mu m^2/100$.

measuring the areas of pores, the overall agreement between the observed and calculated conductances is remarkable.

In figure 3, the number of pores per egg is shown as a function of egg weight. Half of these values are taken from the data of Tullett (1976). The equation describing the allometric relationships between total pore number (N) and egg weight (W) for these data is:

$$N = 1{,}041 \times W^{0.504} \quad (7)$$

$r^2 = .568$, no. = 46.

OXYGEN CONSUMPTION

The data on the mean rate of oxygen consumption of each of the 11 species in this study are plotted versus days before pipping in figure 4. It will be noticed that in nine of the 11 species the rate observed 2 days before pipping is lower than the rate observed 3 days before pipping, and in 10 of the 11 species the rate observed 1 day before pipping is higher than the rate 2 days before pipping. The lower rate, generally observed 2 days before pipping, is called the "preinternal pipping" (pre-IP) rate because the rise occurring 1 day before pipping is assumed (Hoyt, Vleck, and Vleck 1978) to be analogous to that observed in chicken eggs when the embryo breaks into the air cell and begins the transition to pulmonary respiration (= internal pipping). A generalized pattern of oxygen consumption during the 5 days preceding pipping is presented in figure 5. This pattern was obtained by normalizing the data for each species by dividing the observed mean rates (fig. 4) by the pre-IP rate and then calculating the average normalized value for each day. By doing this, the data on each species were given equal weight. Obviously, figure 5 is only

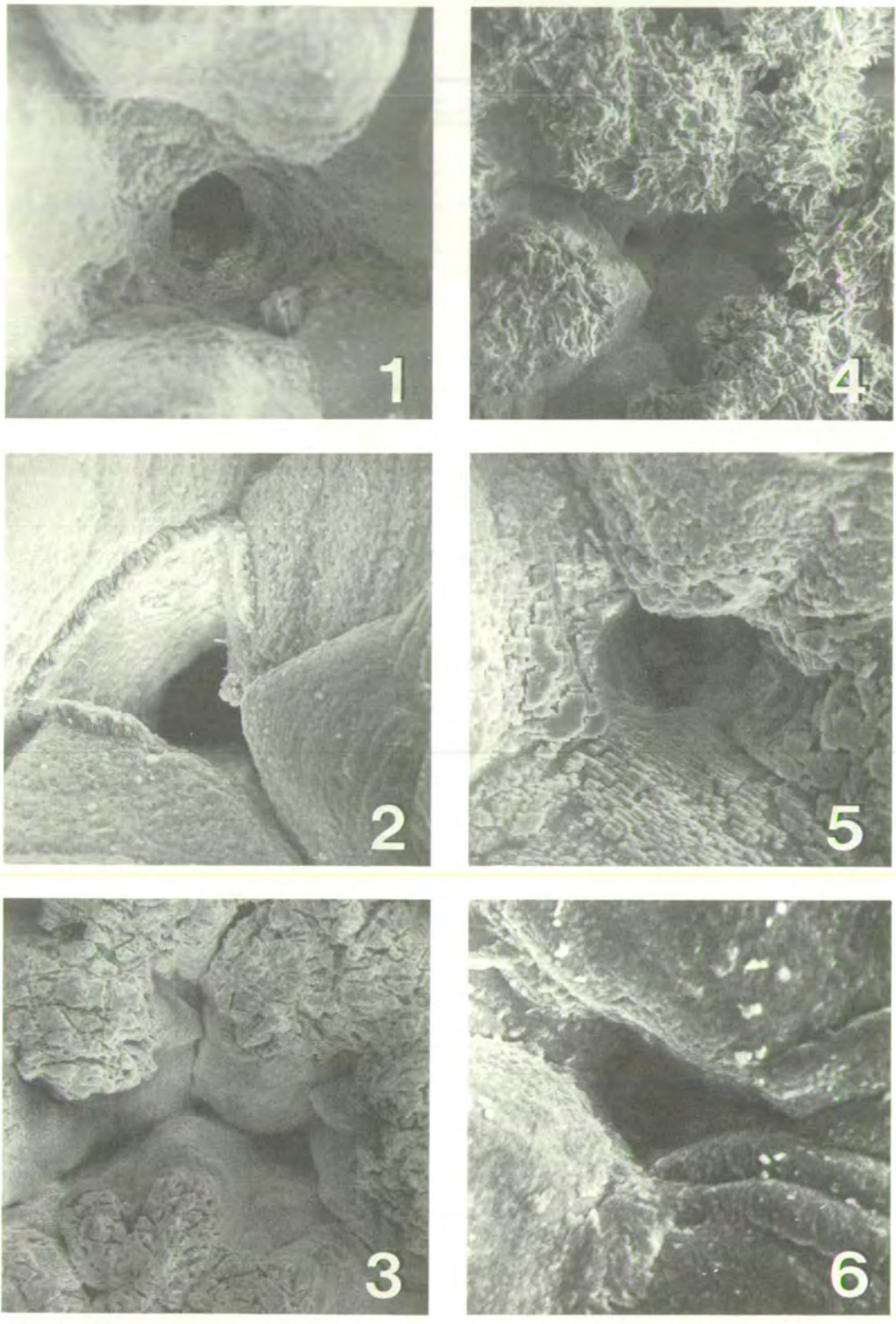

FIG. 1.—Electron micrographs of the inner orifice of the pore canal of six species included in this study. *1*, Abyssinian Blue-winged Goose, *Cyanochen cyanopterus;* × 1,780. *2*, Pink-footed Goose, *Anser brachyrhynchus;* × 1,740. *3*, Hawaiian Goose, *Branta sandvicensis;* × 460. *4*, Emperor Goose, *Anser canagicus;* × 470. *5*, Red-billed Whistling Duck, *Dendrocygna autumnalis;* × 1,950. *6*, Cackling Canada Goose, *Branta canadensis minima;* × 1,950.

cubation period for the eggs of the Anatidae is nearly identical with that of the eggs of birds from 14 other orders.

The conductance coefficient has dimensions of mg $H_2O \cdot torr^{-1} \cdot g^{-1}$. For every 1-torr water-vapor-pressure difference between the inside of the egg and its environment, the conductance coefficient describes the total quantity of water vapor per gram of egg which is lost from the egg during incubation. For example, if during natural incubation of anatid eggs the mean vapor-pressure difference between the inside of the egg and the microclimate of the nest is 30 torr (similar to values found in nonanatid species [Ar and Rahn 1978]), then 5.1×30, or ca. 150 $mg \cdot g^{-1}$, will be lost during the incubation period. This represents 15% of the initial egg weight.

PORE STRUCTURE

Since the gas flux across the eggshell occurs by diffusion (Paganelli et al. 1975), the conductance should be predictable from the number (N), the average cross-sectional area (P_A), and the length (L) of the pores according to equation (3): $G_{H_2O} = 2.24\ (P_A \cdot N)/L$. The geometry of the inner orifice of the pore canals is illustrated in figure 1 for six species. These photographs exemplify the structure of the eggshells included in our study. It can be seen that the pore originates in the valley between the cones. The photograph of the eggshell of the Pink-footed Goose (fig. 1,*2*) is unusual. In this case the pore canal appears to have been initiated in the normal manner, but, at some time in the growth of the shell, the canal was reduced in cross-sectional area by the deposition of a sheet of crystals. In table 2 we have compared the values of conductance calculated with equation (3) with the values observed on the same eggs and presented the values of P_A, N, and L used in the calculation. The percent differences between observed and predicted values of conductance are presented in the last column. The mean difference was −10%, with a standard deviation of 42%. This is similar to the −15% difference in morphological and functional estimates of total pore area (eqq. [5] and [11]) presented by Tullett and Board (1977).

The relatively small difference between the functional and morphometric estimates of G_{H_2O} (table 2) may be considered as a reasonable confirmation of equation (1) and provides additional evidence that the gas flux across an eggshell is a diffusion-limited process. While the overall agreement between functional and morphometric estimates of conductance is good, it is important to recognize that there can be large differences for individual eggs. The average absolute error in our data is 35%, and errors as large as 68% did occur. The major source of error appears to be in the determination of mean pore area. There was considerable variation in the areas of the pores in a single piece of shell. Typically, the standard deviation of the area of the 10 pores was 30% of the mean value. This may reflect real variation in the size of the pores, but it also reflects the fact that determining the area of the funnel-shaped pore orifice is somewhat subjective (see fig. 1).

In figure 2, typical pore casts of anatid eggs published by Tyler (1964) are shown. From the many pores which Tyler pictured, we have selected not more than three for most of the species in table 2 and have drawn them to a common scale. It will be noted that typical anatid pores are not smooth cylinders with parallel walls. When these irregular pore structures are considered, as well as the difficulty encountered in

TABLE 1 (*Continued*)

Tribe and Species	No.	W (g)	G_{H_2O} (mg·Day^{-1}·Torr^{-1})	SD	I (Days)[a]	K_G[b]
Anatini:						
Anas penelope (European Wigeon)	2	36.7	6.1	1.80	24	3.98
A. falcata (Falcated Teal)	5	41.3	7.2	1.27	24	4.15
A. flavirostris (Chilean Teal)	7	28.7	6.0	.39	26	5.48
A. capensis (Cape Teal)	1	30.8	9.2	...	21	6.27
A. gibberifrons gracilis (Australian Grey Teal)	8	32.7	8.5	2.31	24	6.28
A. p. platyrhynchos (Mallard)[d]	11	82.3	14.5	...	28	4.93
A. p. fulvigula (Florida Duck)	3	55.9	16.7	2.15	25	7.47
A. p. diasi (Mexican Duck)	9	46.0	12.2	1.76	27	7.15
A. p. wyvilliana (Hawaiian Duck)	3	50.0	9.5	2.82	27	5.13
A. bahamensis (Bahama Pintail)	1	34.9	8.5	...	25	6.08
A. erythrorhyncha (Red-billed Pintail)	5	37.9	7.5	.47	24	4.75
A. v. versicolor (Versicolor Teal)	3	29.4	4.5	1.26	24	3.67
A. v. puna (Puna Teal)	5	41.8	7.2	2.49	24	4.11
A. discors (Blue-winged Teal)	1	25.4	4.6	...	23	4.22
A. platalea (Red Shoveler)	1	34.9	7.8	...	25	5.56
A. smithi (Cape Shoveler)	1	36.2	7.3	...	26	5.22
Aythyini:						
Netta rufina (Red-crested Pochard)	7	53.7	10.7	1.77	27	5.38
N. peposaca (Rosybill)	2	53.7	15.8	2.36	28	8.23
Aythya americana (Redhead)	1	65.3	13.9	...	24	5.11
A. novae-seelandiae (New Zealand Scaup)	4	63.7	10.5	1.64	26	4.30
Mergini:						
Somateria m. mollissima (European Eider)	1	110.1	19.8	...	26	4.67
S. m. v-nigra (Pacific Eider)[d]	5	100.0	21.4	...	25	5.35
Bucephala albeola (Bufflehead)	1	35.6	5.1	...	30	4.32
B. islandica (Barrow's Goldeneye)	6	67.2	11.4	1.02	32	5.42
B. clangula (Common Goldeneye)	6	64.2	10.6	1.08	30	4.96
Mergus cucullatus (Hooded Merganser)	5	54.8	8.3	2.39	33	4.98
Mergus cucullatus (Hooded Merganser)[e]	5	49.7	6.5	...	31	4.07
M. serrator (Red-breasted Merganser)[e]	10	66.5	6.1	...	29	2.68
M. m. merganser (Goosander)	4	69.3	14.9	2.05	32	6.89
Oxyurini:						
Oxyura jamaicensis (North American Ruddy Duck)	9	74.4	20.3	1.91	21	5.72
O. leucocephala (White-headed Duck)	5	91.6	20.9	3.79	22	5.03
O. vittata (Argentine Ruddy Duck)	3	87.0	22.7	1.69	21	5.47

ductance of the eggs of 90 species from 15 different orders of birds was proportional to the 0.81 power of egg weight. Our data on the eggs of the Anatidae indicate that conductance is proportional to the 0.95 power of egg weight. The reason for this difference is that most of the large eggs in our study are laid by migratory geese, which have relatively short incubation periods and, as would be predicted by Rahn and Ar (1974), have high water-vapor conductances.

Ar and Rahn (1978) have suggested that egg weight (W), water-vapor conductance (G_{H_2O}), and incubation period (I) are interrelated in such a way that the value of $G_{H_2O} \cdot I/W$ is, on average, a constant. They reported a mean value of this "conductance coefficient" ($G_{H_2O} \cdot I/W$) of 5.12 ± 0.86 for 91 species representing 15 orders of birds. We have combined reported values of incubation period with our values of conductance and weight to calculate conductance coefficients for our species (table 1). The mean value of the conductance coefficient for the eggs of the Anatidae is 5.15 ± 1.13. Therefore, the interrelation between conductances, weight, and in-

TABLE 1

WATER-VAPOR CONDUCTANCES (G_{H_2O}) AND CONDUCTANCE COEFFICIENTS (K_G)

Tribe and Species	No.	W (g)	G_{H_2O} (mg · Day^{-1} · Torr^{-1})	SD	I (Days)[a]	K_G[b]
Dendrocygnini:						
Dendrocygna bicolor (Fulvous Whistling Duck)	7	54.4	17.1	1.62	25	7.85
D. arcuata (Wandering Whistling Duck)[c]	4	36.5	6.1	...	30	5.02
D. arborea (Cuban Whistling Duck)	9	59.8	11.6	1.40	30	5.83
D. autumnalis (Red-billed Whistling Duck)	10	42.8	11.6	1.86	27	7.30
Anserini:						
Anser cygnoides (Swan Goose)	7	146.4	26.7	4.70	28	5.11
A. fabalis (Bean Goose)	9	152.3	24.9	7.54	27	4.41
A. f. brachyrhynchus (Pink-footed Goose)	3	139.4	23.4	8.65	27	4.52
A. erythropus (Lesser White-fronted Goose)	7	122.9	20.6	4.75	25	4.19
A. anser (Greylag Goose)	3	162.5	33.2	3.72	27	5.52
A. anser (Graylag Goose)[d]	3	195.0	35.1	...	28	5.04
A. domesticus (Embden Goose)[d]	11	170.0	27.7	...	28	4.56
A. indicus (Bar-headed Goose)	2	110.1	8.4	.66	28	2.14
A. rossi (Ross's Goose)	3	91.8	18.6	1.69	22	4.47
A. canagicus (Emperor Goose)	7	136.1	27.4	5.55	24	4.83
Branta sandvicensis (Hawaiian Goose)	3	154.4	33.4	7.03	30	6.48
B. canadensis leucopareia (Aleutian Canada Goose)	3	116.8	21.4	3.31	28	5.16
B. c. minima (Cackling Canada Goose)	3	100.4	18.0	1.56	28	5.02
B. leucopsis (Barnacle Goose)	7	106.6	19.6	4.57	24	4.41
B. ruficollis (Red-breasted Goose)	5	67.8	12.9	2.70	25	4.74
Tadornini:						
Cyanochen cyanopterus (Abyssinian Blue-winged Goose)	2	82.8	14.7	.88	30	5.33
Chloëphaga picta leucoptera (Greater Magellan Goose)	1	106.1	23.8	...	30	6.72
Chloëphaga poliocephala (Ashy-headed Goose)	1	100.0	13.9	...	30	4.18
Chloëphaga rubidiceps (Ruddy-headed Goose)	3	84.1	11.7	4.11	30	4.16
Tadorna ferruginea (Ruddy Shelduck)	2	79.1	16.6	6.55	29	6.07
T. variegata (Paradise Shelduck)	6	89.6	14.1	1.75	30	4.71
T. tadorna (Common Shelduck)	4	79.9	15.3	1.55	28	5.36
Cairinini:						
Cairina moschata (Muscovy Duck)[d]	4	80.2	12.3	...	35	5.37
C. scutulata (White-winged Wood Duck)	1	99.1	22.8	...	30	6.90
Callonetta leucophrys (Ringed Teal)	10	31.6	6.1	1.75	23	4.41
Aix sponsa (North American Wood Duck)[d]	5	43.3	8.4	...	30	5.82
Aix sponsa (North American Wood Duck)[c]	10	44.4	5.7	...	30	3.86
Aix sponsa (North American Wood Duck)[c]	10	43.4	6.0	...	30	4.15
Anas galericulata (Mandarin Duck)	4	43.1	8.0	1.24	29	5.36
A. galericulata (Mandarin Duck)[c]	2	27.4	3.7	...	29	3.95

NOTE.—No. = sample size; W = fresh egg weight; SD = standard deviation of G_{H_2O}; I = incubation period; $KG = G_{H_2O} \cdot I/W$.

[a] Values obtained from various sources in the literature.

[b] Mean value = 5.15 ± 1.13.

[c] From K. R. Morgan, unpublished data.

[d] From Ar and Rahn 1978.

for the other eggs a volume of 300 cm^3. A single egg was placed in a chamber and immersed in a water bath at 37.5 C. The respiration and compensation chambers were left unsealed to the atmosphere for 15 min to permit thermal equilibration and saturation of the air in both chambers with water vapor. During this period an extra volume of oxygen was injected to make up the metabolic loss. After equilibration the level of the manometer tube connecting the two chambers was kept constant by the displacement of O_2 from a syringe into the respirometer chamber. These volume changes were recorded for 30–60 min until a constant uptake rate was obtained. Values of oxygen consumption were corrected to standard temperature and pressure, dry (STPD).

AIR-CELL GAS TENSIONS

Representative air-cell gas concentrations were obtained by equilibrating over a 24-h period 5 ml of air in a syringe which was attached to an adapter cemented around a hole in the shell over the air cell. Previous analysis has shown that this method gives values comparable to those obtained by direct sampling of the air-cell gases. This new method permits repeated measurements on the same egg on successive days. Gas analysis was done using a 0.5-cm^3 Scholander gas analyzer.

Air-cell gas tensions can also be calculated from the general relationship (Wangensteen and Rahn 1970/1971; Rahn et al. 1974):

$$(P_{I_{O_2}} - P_{A_{O_2}}) = \dot{M}_{O_2}/G_{O_2} \qquad (4)$$

where $P_{I_{O_2}}$ = effective partial pressure of O_2 in the ambient air, 37.5 C, torr; $P_{A_{O_2}}$ = partial pressure of O_2 in the air cell, torr; $\dot{M}_{O_2}$ = daily consumption of egg, ml O_2 (STPD)·day^{-1}; G_{O_2} = O_2 conductance of shell, ml O_2·day^{-1}·$torr^{-1}$ = 1.08 G_{H_2O} (see Appendix).

As discussed by Wangensteen and Rahn (1970/1971), the "effective" ambient O_2 at 37.5 C equals 0.209 (P_B − 48) where 0.209 is the fraction of O_2 in the ambient air, P_B = barometric pressure (torr), and 48 is the vapor pressure of water in the egg at 37.5 C (torr). Substituting and rearranging equation (1), we have:

$$P_{A_{O_2}} = 0.209(P_B - 48) - (\dot{M}_{O_2}/1.08\, G_{H_2O}) . \qquad (5)$$

Assuming that the ambient air has essentially no CO_2 and that during the latter part of incubation the respiratory quotient of the egg is 0.72 (Rahn et al. 1974), one can substitute the $P_{A_{O_2}}$ value into the diffusive-exchange-ratio equation (Wangensteen and Rahn 1970/1971) and obtain:

$$P_{A_{CO_2}} = 0.92\,(P_{I_{O_2}} - P_{A_{O_2}}) . \qquad (6)$$

STATISTICS

All regressions were conducted by the method of least squares, and dependent variables are considered to be significantly correlated with independent variables if the slope of the relation is significantly different from zero ($P < .05$). All sample means are presented with SDs.

RESULTS AND DISCUSSION

WATER-VAPOR CONDUCTANCE

The mean values of water-vapor conductance for the 54 species in this study are presented in table 1. Also included in table 1 are 12 additional values on seven additional species, or subspecies, obtained from the sources indicated. Ar and Rahn (1978) reported that con-

Co., Ltd., Tokyo). Pore counts (pores/cm²) were made on three pieces of shell, one each from the equatorial area and the broad and pointed ends of the egg. The method of counting pores was modified from Tyler (1953); after the cuticle and membranes had been removed by boiling in NaOH, the pores were etched in concentrated nitric acid. The etched piece of shell was then taped to a 35-mm² slide holder and put into a projector. The light transmitted by the pores was focused on a piece of paper 3–4 ft away from the projector. A slide indicating a 1-cm² field was placed in the projector, and the limits of the field were drawn on the piece of paper. Finally, the slide with the piece of shell was put into the projector, and the number of spots of light (= etched pores) falling within the boundary of the 1-cm² field was counted. In some cases this method was not practical because the piece of shell became too fragile before the pores were sufficiently enlarged. In these cases the pores were visualized by the use of methylene blue and were counted under a microscope.

In obtaining a mean pore count (pores/cm²) for the entire egg, the value obtained from the equatorial piece of shell was given twice the weight of the counts obtained from the ends. This was done because pores are not evenly distributed over the shell (Romanoff and Romanoff 1949), and the equatorial piece was taken to represent approximately twice as large a portion of the shell as either of the end pieces. The total number of pores per egg was obtained by multiplying the mean pore count by the total area of the egg estimated from volume with equation (12) of Hoyt (1976).

CALCULATION OF WATER-VAPOR CONDUCTANCE FROM PORE DIMENSIONS

One should be able to calculate the water-vapor conductance from the pore geometry of the shell using Fick's first law (Rahn et al. 1976):

$$G_{H_2O} = (A_p/L)(D_{H_2O}/RT)(6.94 \cdot 10^3), \quad (1)$$

where G_{H_2O} = water vapor flux per torr, mg·day^{-1}·torr^{-1}; A_p = total effective pore area, mm²; L = length of pore or shell thickness, mm; D_{H_2O} = diffusion coefficient of water vapor, cm²·sec^{-1}; RT = gas constant times absolute temperature, torr; and 6.94×10^3 = constant to adjust units on both sides of the equation.

The constants $(D_{H_2O}/RT)(6.94 \times 10^3)$ can be reduced to the single value 2.24. Since

$$A_p = P_A \cdot N \quad (2)$$

where P_A = cross-sectional area of single pore, mm², and N = total number of pores per egg, we can substitute equation (2) and the constants into equation (1) to obtain the following relationship:

$$G_{H_2O} = (2.24\, P_A \cdot N)/L, \quad (3)$$

which allows one to predict the conductance from the dimensions P_A, L, and N.

OXYGEN CONSUMPTION

Sixty-four eggs from 11 species of ducks, geese, and swans were incubated at 37.5 C. Fresh egg weight was estimated from length, breadth, and data from Schönwetter (1960–1961), using the method of Hoyt (1979).

The rate of oxygen consumption was determined in a modified volumetric compensated microrespirometer (Scholander 1950). The chambers used for the two largest species of eggs had an internal volume of 900 cm³ and those used

pressure of CO_2 increases (Wangensteen and Rahn 1970/1971). It is these internal partial pressures with which the physiology of the embryo must deal. The balance between O_2 consumption and CO_2 production is such that all weight loss of the egg is due to the loss of water. The rate of water loss is proportional to the 0.75 power of the egg weight (Drent 1970), and a total of about 16% of the fresh egg weight is lost during the course of incubation (Drent 1975, p. 351) and is presumably optimal for hatching success.

Therefore, the gas conductance of the shell must be a compromise between achieving the proper weight loss and permitting the necessary respiratory exchange; and gas conductance, metabolic rate, pore geometry, and air-cell gas tensions are all necessarily interrelated. Shell conductance (Ar et al. 1974; Ar and Rahn 1978) and pore morphology (Tullett 1975; Board, Tullett, and Perrott 1977) have been studied in a wide variety of avian species. The rate of oxygen consumption and air-cell gas tensions (Rahn, Paganelli, and Ar 1974) have been determined for a smaller number of species. However, all of these studies have been broad surveys, and for only a few domestic species have all four parameters been determined.

We have studied gas conductance, pore morphology, oxygen consumption, and air-cell gas tensions in the eggs of the family Anatidae, which permits us to provide a well-integrated description of the eggs of a closely related group of birds. Our results are remarkably similar to those obtained on much more diverse groups of bird eggs and support the general applicability of the descriptions provided by these broad surveys.

MATERIAL AND METHODS

WATER-VAPOR CONDUCTANCE

Water-vapor conductance (G_{H_2O}) was determined on a total of 255 eggs from 54 species of ducks and geese obtained at the Wildfowl Trust, Slimbridge, England. These eggs had been removed from their nests, incubated for 7–10 days by bantam hens, and shown, by candling, to be infertile. They were then placed in desiccators with silica gel at 25 C, and the rate of weight loss was determined by weighing them daily for 6 days. Previous experience has shown that the weight loss during the first day of desiccation is usually significantly higher than the average value during succeeding days. For this reason, the weight loss during the first day was not included in the calculation of G_{H_2O}. The conductance was computed by dividing the average daily weight loss by 23.7 torr (the saturation water-vapor pressure in the egg at 25 C) and making the appropriate correction for the barometric pressure (Ar et al. 1974). The value reported is the average of four values determined on 4 consecutive days.

PORE STRUCTURE

Shell thickness and the area of individual pores were determined by the methods of Tullett and Board (1977). Thickness was measured on 10 pieces of shell taken from randomly selected areas of the egg. Pore area was measured on 10 pores selected from a single piece of shell taken from the equatorial area of the egg after the membranes had been removed by boiling in 5% NaOH. The inner surface was examined by scanning electron microscopy, and tracings of the outlines of the pores were made on tracing paper placed on the viewing screen. These tracings were then cut out, and their areas were measured with an automatic area meter (Hayashi Denko

THE EGGS OF THE ANATIDAE: CONDUCTANCE, PORE STRUCTURE, AND METABOLISM[1]

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The water-vapor conductance (G_{H_2O}) of the eggs of 61 species of birds of the family Anatidae is reported. The relationship between egg weight (W), incubation period (I), and conductance for these species is similar to that of the eggs of 15 orders of birds; the mean value of the conductance coefficient ($G_{H_2O} \cdot I/W$) is 5.15 ± 1.13. Pore area, using electron microscopy, pore length, and the total number of pores per egg were measured to calculate water-vapor conductance according to Fick's law of diffusion. The mean difference between the calculated value and the value directly measured on the same egg was −10%. The rate of oxygen consumption near the end of incubation was determined for the eggs of 11 species. In general, the rate observed 2 days before pipping is lower than the rate observed 3 days and 1 day before pipping. The allometric relationship between egg weight and the rate of oxygen consumption ($\dot{M}_{O_2}$) 2 days before pipping is proportional to the 0.72 power of egg weight. The air-cell gas tensions prior to pipping were either measured directly or calculated from the O_2 conductance and O_2 consumption. The mean partial pressures for O_2 and CO_2 for 11 species were 103 and 41 torr, respectively. These functional properties of Anatid eggs appear to fall in line with those described for bird eggs of taxonomically more diverse groups.

INTRODUCTION

The shell of an avian egg limits the gaseous exchange between the inside of the egg and its environment (Kutchai and Steen 1971; Wangensteen and Rahn 1970/1971). The exchange takes place by diffusion through gas-filled pores (Paganelli et al. 1975; Paganelli, Ackerman, and Rahn 1978), and it is the number and structure of these pores which determine the gas conductance of the egg and limit the rate of diffusion. Water vapor, O_2, and CO_2 are the primary substances exchanged (Drent 1970), but their rates of exchange differ. Water is lost at a constant rate (Drent 1970), but the metabolic rate of the embryo increases progressively throughout incubation. Since the gas conductance is fixed after the first half of incubation (Kutchai and Steen 1971; Wangensteen and Rahn 1970/1971; Lomholt 1976*a*; Tullett and Board 1976), the partial pressure of O_2 inside the shell decreases and the partial

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Water Vapour Conductance of Wildfowl Eggs and Incubator Humidity

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Introduction

Fertile eggs lose water progressively throughout incubation, the loss being a function of the water vapour concentration in the atmosphere around the eggs, eggshell porosity, temperature and the length of incubation. Optimum hatchability of domestic hen eggs occurs when about 12% of the initial weight of an egg is lost before pipping, and 4% between pipping and hatching (Lundy,1969; Robertson, 1961a,b).Weight loss is due entirely to the diffusion of water vapour from the egg contents through the pores in the eggshell (Romanoff & Romanoff, 1949). Field observations have shown also a 12% weight loss (Drent, 1975); a few exceptions have been noted. This level of loss of fresh egg weight is common to all sizes of egg, the actual rate appears to be adapted to incubation period (Rahn & Ar, 1974; Ar & Rahn, 1978). Thus the eggs of birds with prolonged incubation, e.g. Shearwaters (Whittow, 1980) and Kiwis (Calder, 1978), still lose only 12% weight.Indeed, adaption of the eggshell porosity results in a constant egg weight loss across the broad spectrum of egg size and variation in incubation period (Rahn & Ar, 1974). There is no appreciable water loss from the eggs of the Brush Turkey, Alectura lathami (Seymour & Rahn, 1978) and the Mallee Fowl, Leipoa ocellata (Seymour & Ackerman, 1980) due to incubation in mounds having saturated atmospheres.

To achieve a 12% loss in the eggs of domesticated species the humidity settings required for artificial incubators used in the poultry industry have been established empirically over many years (Lundy, 1969). Such an approach is possible when large

numbers of eggs are available for such a trial and error approach it cannot be considered by aviculturists who often have few eggs and a need for a high hatchability, especially with birds whose existence in the wild is threatened. In the absence of pertinent information, they have adopted generally the humidities which have been selected for domesticated species on the unwarranted assumption that such values are suitable for the eggs of wild birds also.

Recent advances in our understanding of the mechanism of water loss from incubating eggs means that it is now possible to predict the humidity setting for an incubator that will ensure a 12% weight loss in the egg(s) of a given species (Tullett, 1981). There are two possible approaches to establishing the humidity setting required in an artificial incubator. In one case, the humidity in the nest cup of the species in question can be determined with an egg hygrometer - an eggshell filled with silica gel (Rahn et al., 1977), or with electronic sensors (Howey et al., 1977). These techniques will indicate also whether or not there are changes in nest humidity during incubation. In the other case measurements of the porosity of the eggshell are used to calculate the required incubator humidity which is needed to achieve 12% fresh egg weight loss (Tullett, 1981). This present communication, which involved the second approach, discusses the probable incubator humidity settings for 36 species of Anatidae.

Mechanism of water loss - the theory

Rahn and his collegues (Rahn & Paganelli, 1981) have shown that water is lost from eggs by diffusion of vapour through pores in the eggshell (Fig. 1), the loss being governed by Fick's law of diffusion (Wangensteen & Rahn, 1970/71). It can be shown that (Ar et al., 1974):

$$\dot{M}_{H_2O} = G_{H_2O} \cdot \Delta P_{H_2O} \qquad (1)$$

$\dot{M}_{H_2O}$ is the rate of water loss per day (mg/day); G_{H_2O} is the water vapour conductance of the eggshell (mg/day/torr), - i.e. a measure of the potential porosity of the eggshell to water vapour - which is determined by the number and the geometry of the pores in the eggshell and the diffusivity of water vapour. ΔP_{H_2O} (torr*) is the difference between the water vapour partial pressure inside (P_{H_2O} egg) and outside (P_{H_2O} nest) of the egg, where P_{H_2O} egg has been shown to be saturated at incubation temperatures. As it is a direct measure of water vapour concentration, water vapour partial pressure is used in preference to the more commonly used term, relative humidity. These two are related thus (Unwin, 1980):

$$R.H.\% = \frac{P}{P(sat.)} \times 100 \qquad (2)$$

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* In this paper partial pressure is measured in torr as the unit in general use in egg respiration physiology. Millibars is the unit in the S.I. convention. These units are related thus:

760 torr = 1013 millibars 0.75 torr = 1 millibar

where P = measured partial pressure (torr); P(sat.) = saturated partial pressure at incubator temperature (torr), these values can be obtained from standard tables (e.g Unwin, 1980).

Oxygen and carbon dioxide diffusion across the eggshell can be described in the same manner as for water vapour. Equation (1) can be generalized to describe any gas diffusing across a shell:

$$\dot{M}_x = G_x \cdot \Delta P_x \qquad (3)$$

where $\dot{M}_x$ = rate of flux of gas x (ml/day); G_x = the conductance of the shell to gas x (ml/day/torr); ΔP_x = the difference in partial pressure of gas x across the shell (torr). There is a linear relationship between G_{H_2O}, G_{O_2} (oxygen conductance), and G_{CO_2} (carbon dioxide conductance), and it is possible to calculate G_{O_2} and G_{CO_2} from G_{H_2O} (Hoyt et al., 1979)

If the G_{H_2O} of an eggshell is known then the correct incubator humidity setting to achieve a 12% fresh egg weight loss during incubation can be calculated from equation (1). The aggregate 12% weight loss can be used to establish the daily weight loss requirement, viz:

$$\dot{M}_{H_2O} = \frac{W \times 0.12 \times 1000}{(I - 2)} \qquad (4)$$

where W = fresh egg weight (grams); (I -2) = incubation period minus 2 days for the hatching period (days); the equation is multiplied by 1000 to convert grams into milligrams. Incubator partial pressure can be calculated rearranging equation (1),viz:

$$P_{H_2O\ nest} = P_{H_2O\ egg} - (\dot{M}_{H_2O}/G_{H_2O})$$

P_{H_2O} egg is obtained by consulting standard tables (e.g. Unwin, 1980) of saturated water vapour partial pressure at incubation temperature.

G_{H_2O} can be determined by storing an egg in an environment where P_{H_2O} is known and daily weight loss ($\dot{M}_{H_2O}$) is measured. With an egg in a desiccator, having a P_{H_2O} = zero, then ΔP_{H_2O} = P_{H_2O} egg, and $G_{H_2O} = \dot{M}_{H_2O} / P_{H_2O}$ egg (Ar et al., 1974).

Materials and methods

Three hundred and fifty eggs from 76 species of Anatidae and 1 species of Phoenicopteridae were obtained from the Wildfowl Trust, Slimbridge, England in the 1980 and 1981 breeding seasons. The eggs had shown no embryo development during incubation for six days under bantam hens.

In the 1980 breeding season, the water vapour conductance of all eggs were determined as in Hoyt et al. (1979).They were stored in desiccators containing silica gel at 25°C, and weighed daily for up to seven days. The silica gel was replenished regularly.

To overcome the potential problem of the back pressure of water vapour in the desiccator (where desiccator partial pressure no longer equals zero; Dr. A. H. J. Visschedijk, pers. comm.), the Hoyt et al. (1979) technique was modified for the 1981 breeding season. The air in the desiccators was circulated by an aquarium pump to ensure rapid uptake of water vapour by the silica gel.

The water vapour conductance was calculated by dividing the average daily weight loss for each egg by the saturation water vapour partial pressure in the egg at 25 °C (23.7 torr). The result was corrected for barometric pressure (Ar et al., 1974), obtained from the Gloucester Meterological Office, Gloucester, approximatly 15 miles from Slimbridge.

Results and discussion

The water vapour conductances (G_{H_2O}) obtained in this study, as well as those quoted in the literature, for Anatidae eggs are summarised in Table 1. Fresh egg weights and incubation periods were obtained from the literature or records at the Wildfowl Trust.

The calculated incubator humidity required for 36 species from 9 tribes of Anatidae are shown in Table 2. It has been assumed that, with an incubator temperature of 37°C, 12% fresh egg weight should be lost during the incubation period up until pipping (normally 2 - 3 days before hatching). The nest humidity of only a few Anatidae have been measured (Table 3). It is noteworthy, however, that the reported values are similar to those predicted in Table 2.

It is evident from Table 2 that the estimated relative humidity for an artificial incubator set at 37°C covers a broad spectrum, viz 0 - 70% R.H. With most of the Anatidae eggs, 0 - 50% R.H. would assure a 12% loss in egg weight during incubation.The eggs of the Whistling Ducks (Dendrocygnini) and the White-winged Wood Duck (Cairina scutulata), however, appear to require incubator humidities of around 60% and 70% R.H. respectively. The Red-breasted Merganser (Mergus s. serrator), on the other hand, cannot lose 12% of its fresh egg weight even with an incubator humidity of 0% R.H..

Most of these suggested settings contrast with the normal (70% R.H.) that is commonly used in artificial incubation. Morover our results suggest that several incubators set at

different humidities are probably required to incubate successfully a range of Anatidae eggs. There would appear to be a need for an improvement in incubator design, simply because the general range of incubators used in aviculture do not permit easy maintenance of a defined humidity.

It should also be noted that eggs that require low incubator humidities also require higher ventilation rates, particuarly just prior to pipping. Such eggs have a low G_{H_2O}, and therefore a low G_{O_2} and G_{CO_2}. To ensure sufficient oxygen flux into the egg to meet the embryo's metabolic demand, and rapid removal from the egg of carbon dioxide to prevent asphyxiation, ΔP_{O_2} and ΔP_{CO_2} must be increased (equation 3). This can be achieved by increasing incubator ventilation. Porosity may be so reduced in certain eggs that the embryo's requirements at the late stage of incubation will not be met even with increased ventilation.

Table 1 shows that there are large variations in G_{H_2O} within a species. Therefore in instances where it is important to try to maximise a hatch - as in the case of the eggs of rare Wildfowl - it would be preferable to determine the required humidity for each egg by measuring its G_{H_2O} before the onset of incubation, rather than taking the values cited in this paper. Tullett (1981) described methods for this purpose; he used eggs with a known G_{H_2O} to establish the G_{H_2O} of other eggs. The G_{H_2O} of the 'calibrated' egg - this can be any egg - can be determined by the methods described above. The 'calibrated' egg is then stored with the other of unknown G_{H_2O} and weight loss recorded daily. The unknown G_{H_2O} can be calculated, viz:

$$\frac{\dot{M}_{H_2O}\,(\text{calibrated egg})}{G_{H_2O}\,(\text{calibrated egg})} = \frac{\dot{M}_{H_2O}\,(\text{unknown egg})}{G_{H_2O}\,(\text{unknown egg})}$$

The daily weighings need to be repeated until a constant G_{H_2O} is calculated, normally after 2 - 3 days. The only expensive equipment required is a balance, accurate to 0.01 grams for small eggs (<50 grams). With less accurate balances, it may be possible to weigh the whole clutch and determine the mean daily egg-weight loss. This would be a reasonably accurate method as G_{H_2O} varies less within a clutch than between cluthes (Sotherland et al., 1979).

The eggs of the Red-breasted Merganser (Mergus s. serrator) deserve special mention. In this study as well as that of Morgan (unpublished observations), their G_{H_2O} was very low (Table 1). There is some evidence that the G_{H_2O} of unincubated Anatidae eggs is lower than incubated ones (Prof. H. Rahn, pers. comm.), and the Red-breasted Merganser eggs used in this study were unincubated. It is not known where Morgan obtained his/her eggs nor whether or not they were incubated. Passerine eggshells have been shown to increase their G_{H_2O} at the onset of incubation (Carey, 1979), but the evidence for this change in G_{H_2O} in Anatidae eggs is tentative, and needs further study. If shown to be true, it will dictate that G_{H_2O} determination in hatcheries should occur after the onset of incubation.

Attention needs to be directed to several practices in aviculture which may well need to be reconsidered. Avicultural practice ought to ensure that an established stock of captive breeding birds is not subjected to selection pressures such that

there is a marked change in the G_{H_2O} of the eggshell. Not only would such eggs cause a progressive reduction in hatchability, but adjusting incubator conditions such that only a few eggs produced hatchlings could be expected to favour those birds having traits for abnormal shell formation. Such birds would be unsatisfactory sources of eggs or young, for re-establishment of the species in the wild. Thus measurement of shell G_{H_2O} ought to be done routinely on the eggs of captive species and the selection programme adjusted accordingly. It would also appear to be reasonable to assume that measurement of the G_{H_2O} of eggs collected in the wild ought to be determined before progeny are introduced into bird collections.

The eggshell G_{H_2O} of captive birds must then be maintained at a norm through many generations. This requires the eggs to be incubated at the **correct humidity** to prevent a selection of eggs with abnormal water vapour conductance.

Summary

The humidity setting for an artificial incubator for the eggs of most avian species can be assessed by measuring the water vapour conductance (G_{H_2O}) of the eggshell. This study reports the G_{H_2O} from 350 eggs of 76 species of Anatidae and 1 species of Phoenicopteridae. These data, combined with G_{H_2O} of Anatidae eggs reported in the literature, were used to estimate the required incubator humidity setting for 36 Anatidae species. Techniques for measuring eggshell G_{H_2O} in a hatchery are proposed, and the implications of G_{H_2O} for avicultural practice are discussed.

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References

Ar, A., Paganelli, C.V., Reeves, R.B., Greene, D.G., & Rahn, H. 1974. The avian egg: water vapour conductance, shell thickness and functional pore area. Condor 76: 153-158.

Ar, A., & Rahn, H. 1978. Interdependence of gas conductance, incubation length and weight of the avian egg. Pps 227-236 Piiper J. (ed.) Respiratory function in birds, adult and embryonic. Springer-Verlag, New York.

Calder, W.A. III 1978. The kiwi: a case of compensating divergences from allometric predictions. Pps 239-242 Piiper, J. (ed.) Respiratory function in birds, adult and embryonic. Springer-Verlag, New York.

Carey, C. 1979. Increase in conductance to water vapour during incubation in eggs of two avian species. J. of Exp. Zool. 209: 181-186.

Drent, R.H. 1975. Incubation. Pps 333-420 Farner, D.S. & King, J.R. (eds.) Avian Biology. Vol. 5. Academic Press, New York.

Howey, P.W. 1982. A radio telemetric study of the avian nest microclimate. Ph.D. Thesis. University of Bath.

Howey, P.W., Board, R.G. & Kear, J. 1977. A pulse-position-modulated multichannel radio telemetry system for the study of the avian nest microclimate. Biotelemetry 4: 169-180.

Hoyt, D.F., Board, R.G., Rahn, H. & Paganelli, C.V. 1979. The eggs of the Anatidae: Conductance, pore structure and metabolism. Physiol. Zool. 52: 438-450.

Lundy, H. 1969. A review of the effects of temperature, humidity, turning and gaseous environment in the incubator on the hatchability of the hens egg. Pps 143-176 Carter T.C. & Freeman, B.M. (eds.) The fertility and hatchabilty of the hen's egg. Oliver & Boyd, Edinburgh.

Rahn, H., Ackerman, R.A. & Paganelli, C.V. 1977. Humidity in the avian nest and egg water loss during incubation. Physiol. Zool. 50: 269-283.

Rahn, H. & Ar, A. 1974. The avian egg: incubation time and water loss. Condor 76: 147-152.

Rahn, H. & Paganelli, C.V. 1981. Gas exchange in the avian egg. State University of New York, Buffalo.

Robertson, I.S. 1961a. Studies on the effects of humidity on the hatchability of the hen's egg. I. The determination of optimum humidity. J. of Argric. Sci., Cambs. 57: 185-194.

Robertson, I.S. 1961b. Studies on the effects of humidity on the hatchability of the hen's egg. II. A comparison of hatchability, weight loss and embryonic growth in eggs incubated at 40 and 70% R.H. J. of Agric. Sci., Cambs. 57: 195-198.

Romanoff, A.L. & Romanoff, A.J. 1949. The avian egg. John Wiley & Sons, New York.

Seymour, R.S. & Ackerman, R.A. 1980. Adaptions to underground nesting in birds and reptiles. Amer. Zool. 20: 437-447.

Seymour, R.S. & Rahn, H. 1978. Gas conductance in the eggshell of the mound-building brush turkey. Pps 243-246 Piiper, J. (ed) Respiratory function in birds, adult and embryonic. Springer-Verlag, New York.

Snyder, G.K., Black, C.P. & Birchard, G.F. 1982. Development and metabolism during hypoxia in embryos of high altitude Anser indicus verses sea level Branta canadensis geese. Physiol. Zool. 55: 113-123.

Sotherland, P.R., Packard, G.C. & Taigen, T.L. 1979. Permiability of magpie and blackbird eggshells to water vapour: variation amoung and within nests of a single population. Auk 96: 192-195.

Tullett, S.G. 1981. Theoretical and practical aspects of eggshell porosity. Turkey 29: 24-28.

Unwin, D.M. 1980. Microclimate measurement for ecologists. Academic Press, London.

Wangensteen, O.D. & Rahn, H. 1970/71. Respiratory gas exchange by the avian embryo. Respir. Physiol. 11: 31-45.

Whittow, G.C. 1980. Physiological and ecological correlates of prolonged incubation in sea birds. Amer. Zool. 20: 427-436.

Table 1. The water vapour conductances (G_{H_2O}), fresh egg weight (W) and incubation period (I) for Anatidae eggs. Figures in brackets are standard deviations.

SPECIES	No.	G_{H_2O} (mg/day/torr)	W (gm)	I (day)
Anatidae				
Dendrocygnini				
Dendrocygna				
guttata (Spotted Whistling Duck)	3	11.76 (2.80)	50	30.5
bicolor (Fulvous Whistling Duck)	20	14.27 (2.51)	51	26.0
bicolor (Fulvous Whistling Duck)a	7	17.1 (1.62)	54	25.0
arborea (Cuban Whistling Duck)	6	18.47 (4.26)	54	30.0
arborea (Cuban Whistling Duck)a	9	11.6 (1.40)	60	30.0
arcuata (Wandering Whistling Duck)b	4	6.1	36	30.0
viduata (White-faced Whistling Duck)	9	8.35 (0.56)	36	27.0
autumnalis (Red-billed Whistling Duck)	10	11.6 (1.86)	43	27.0
Thalassornis				
l. leuconotus (African White-backed Duck)	1	21.79	84	26.0
Anserini				
Cygnus				
melanocoryphus (Black-necked Swan)	5	41.94 (6.87)	247	36.0
columbianus bewickii (Bewick's Swan)	4	38.81 (2.54)	260	29.5
Anser				
cygnoides (Swan Goose)a	7	26.7 (4.70)	146	28.0
fabalis (Bean Goose)a	9	24.9 (7.54)	152	27.0
fabalis rossicus (Russian Bean Goose)	2	33.67 (0.42)	146	28.0
fabalis brachyrhynchus (Pink-footed Goose)a	3	23.4 (8.65)	139	27.0
albifrons frontalis (Pacific Whitefront)	6	23.32 (5.66)	133	26.0
albifrons gambelli (Tule Goose)	1	22.27	133	26.5
albifrons flavirostris (Greenland Whitefront)	1	15.36	117	26.5
erythropus (Lesser Whitefront)	3	25.10 (4.03)	100	25.0
erythropus (Lesser Whitefront)a	7	20.6 (4.75)	123	25.0
canagicus (Emperor Goose)	6	23.06 (4.45)	120	24.5
canagicus (Emperor Goose)a	7	27.4 (5.55)	136	24.0
anser (Greylag Goose)a	3	33.2 (3.72)	163	27.0
anser (Greylag Goose)c	3	35.1	195	28.0
domesticus (Embden Goose)c	11	27.7	170	28.0
indicus (Bar-headed Goose)a	2	8.4 (0.66)	110	28.0
indicus (Bar-headed Goose)d	?	25.6	110	28.0
coerulescens atlanticus (Greater Snow Goose)	5	25.15 (1.46)	127	23.5
rossi (Ross's Goose)	3	18.6 (1.69)	92	22.0
Branta				
canadensis parvipes (Lesser Canada Goose)	5	36.30 (2.48)	91	24.5
canadensis leucopareia (Aleutian Canada Goose)	5	23.58 (3.80)	93	27.5
canadensis leucopareia (Aleutian Canada Goose)a	3	21.4 (3.31)	117	28.0
canadensis minima (Cackling Canada Goose)a	3	18.0 (1.56)	100	28.0
canadensis (Canada Goose)d	?	38.0	...	....

sandvicensis (Hawaiian Goose)	2	33.68	(4.10)	131	29.0
sandvicensis (Hawaiian Goose)a	3	33.4	(7.03)	154	30.0
leucopsis (Barnacle Goose)	15	19.76	(5.19)	107	24.5
leucopsis (Barnacle Goose)a	7	19.6	(4.57)	107	24.0
ruficollis (Red-breasted Goose)	1	5.77		90	24.0
ruficollis (Red-breasted Goose)a	5	12.9	(2.70)	68	25.0
Tadornini					
Tadorna					
tadorna (Common Shelduck)	4	10.84	(5.66)	78	30.0
tadorna (Common Shelduck)a	4	15.3	(1.55)	80	28.0
variegata (Paradise Shelduck)	2	9.71	(5.46)	91	30.0
variegata (Paradise Shelduck)a	6	14.1	(1.75)	90	30.0
ferruginnea (Ruddy Shelduck)	2	11.72	(0.83)	83	28.0
ferruginnea (Ruddy Shelduck)a	2	16.6	(6.55)	79	29.0
Cyanochen					
melanoptera (Abyssinian Blue-winged Goose)	5	16.17	(4.60)	85	32.0
melanoptera (Abyssinian Blue-winged Goose)a	2	14.7	(0.88)	83	30.0
Neochen					
jubatus (Orinoco Goose)	8	10.65	(0.75)	63	30.0
Chloephaga					
poliocephala (Ashy-headed Goose)	5	6.55	(1.47)	89	30.0
poliocephala (Ashy-headed Goose)a	1	13.9		79	30.0
picta picta (Lesser Magellan Goose)	2	19.63	(5.07)	122	30.0
picta leucoptera (Greater Magellan Goose)a	1	23.8		106	30.0
rubidiceps (Ruddy-headed Goose)a	3	11.7	(4.11)	84	30.0
Anatini					
Marmaronetta					
angustirostris (Marbled Teal)	1	9.41		31	25.0
Anas					
versicolor versicolor (Northern Versicolour Teal)	8	6.11	(0.77)	34	25.5
versicolor versicolor (Northern Versicolour Teal)a	3	4.5	(1.26)	29	24.0
versicolor puna (Puna Teal)	6	7.88	(0.85)	42	24.0
versicolor puna (Puna Teal)a	5	7.2	(2.49)	42	24.0
erythrorhyncha (Red-billed Pintail)	2	11.84	(0.23)	39	26.0
erythrorhyncha (Red-billed Pintail)a	5	7.5	(0.47)	38	24.0
acuta acuta (Northern Pintail)	1	3.64		45	23.5
bahamensis (Bahama Pintail)a	1	8.5		35	25.0
crecca carolinensis (American Green-winged Teal)	2	2.63	(0.63)	?	21.0
falcata (Falcated Teal)a	5	7.2	(1.27)	41	24.0
flavirostris (Chilean Teal)a	7	6.0	(0.39)	29	26.0
capensis (Cape Teal)a	1	9.2		31	21.0
gibberifrons gracilis (Australian Grey Teal)	9	6.94	(2.53)	35	24.5
gibberifrons gracilis (Australian Grey Teal)a	8	8.5	(2.31)	33	24.0
castanea (Chestnut Teal)	4	11.61	(1.23)	40	28.0
aucklandica chlorotis (New Zealand Brown Teal)	7	16.75	(1.21)	62	28.5
platyrhynchos platyrhynchos (Mallard)	4	11.35	(6.35)	54	28.0
platyrhynchos platyrhynchos (Mallard)c	11	14.5		82?	28.0
platyrhynchos diasi (Mexican duck)	1	10.72		58	27.0
platyrhynchos diasi (Mexican Duck)a	9	12.2	(1.76)	46	27.0

platyrhynchos fulvigula (Florida Duck)a	3	16.7 (2.15)	56	25.0
platyrhynchos wyvilliana (Hawaiian Duck)a	3	9.5 (2.82)	50	27.0
luzonica (Philippine Duck)	3	13.77 (2.12)	51	25.5
poecilorhyncha poecilorhyncha (Indian Spotbill)	1	9.30	57	28.0
poecilorhyncha zonorhyncha (Chinese Spotbill)	1	12.97	?	?
melleri (Meller's Duck)	2	16.20 (3.14)	?	?
sparsa sparsa (African Black Duck)	2	10.02 (0.04)	72	28.0
penelope (Wigeon)	4	5.45 (1.32)	44	24.0
penelope (Wigeon)a	2	6.1 (1.80)	37	24.0
americana (American Wigeon)	2	7.22 (0.25)	43	24.5
sibilatrix (Chiloe Wigeon)	1	7.88	53	26.0
discors (Blue-winged Teal)a	1	4.6	25	23.0
rhynchotis rhynchotis (Australian Shoveler)	1	4.91	43	26.0
platalea (Red Shoveler)a	1	7.8	35	25.0
smithi (Cape Shoveler)a	1	7.3	36	26.0
Merganettini				
Merganetta				
armata armata (Chilean Torrent Duck)	3	10.43 (0.93)	?	?
Somateriini				
Somateria				
mollissima mollissima (European Eider)	8	21.38 (5.98)	110	26.5
mollissima mollissima (European Eider)a	1	19.8	110	26.0
mollissima v-nigra (Pacific Eider)c	5	21.4	100	25.0
spectabilis (King Eider)	1	21.48	73	23.0
fischeri (Spectacled Eider)	5	18.35 (4.78)	73	24.0
Aythyini				
Netta				
rufina (Red-creasted Pochard)	1	7.71	56	27.0
rufina (Red-creasted Pochard)a	7	10.7 (1.77)	54	27.0
peposaca (Rosybill)a	2	15.8 (2.36)	54	28.0
Aythya				
valisineria (Canvasback)	2	15.89 (0.45)	68	24.5
nyroca (Ferruginous Duck)	1	8.38	43	26.0
baeri (Baer's Pochard)	2	4.75 (2.24)	43	27.0
americana (Redhead)a	1	13.9	65	24.0
novoe-seelandioe (New Zealand Scaup)	4	14.20 (7.08)	63	28.5
novoe-seelandioe (New Zealand Scaup)a	4	10.5 (1.64)	64	26.0
fuligula (Tufted Duck)	8	9.09 (1.70)	56	24.0
affinis (Lesser Scaup)	8	7.99 (3.31)	51	24.0
marila mariloides (Pacific Greater Scaup)	1	13.70	67	24.5
Cairinini				
Calonetta				
leucophrys (Ringed Teal)	20	5.59 (1.98)	32	27.0
leucophrys (Ringed Teal)a	10	6.1 (1.75)	32	23.0
Chenonetta				
jubata (Australian Wood Duck)	1	7.11	54	28.0
Aix				
galericulata (Mandarin)	8	4.43 (1.77)	41	29.0
galericulata (Mandarin)a	4	8.0 (1.24)	43	29.0
galericulata (Mandarin)b	2	3.7	27	29.0
sponsa (North American Wood Duck)	1	3.87	44	30.0
sponsa (North American Wood Duck)c	5	8.4	43	30.0
sponsa (North American Wood Duck)b	10	5.7	44	30.0

sponsa (North American Wood Duck)b	10	6.0		43	30.0
Sarkidiornis					
melanotos melanotos (Comb Duck)	10	8.26	(0.79)	66	30.0
Cairina					
moschata (Muscovy Duck)	9	11.86	(2.15)	74	35.0
moschata (Muscovy Duck)c	4	12.3		80	35.0
scutulata (White-winged Wood Duck)	12	19.88	(5.60)	72	34.0
scutulata (White-winged Wood Duck)a	1	22.8		99	30.0
Mergini					
Bucephala					
islandica (Barrow's Goldeneye)	9	8.60	(2.11)	70	32.0
islandica (Barrow's Goldeneye)a	6	11.4	(1.02)	67	32.0
clangula clangula (Goldeneye)	8	10.59	(2.96)	57	30.0
clangula clangula (Goldeneye)a	6	10.6	(1.08)	64	30.0
Mergus					
albellus (Smew)	14	9.07	(1.51)	42	28.0
cucullatus (Hooded Merganser)	5	7.40	(3.80)	60	32.5
cucullatus (Hooded Merganser)a	5	8.3	(2.39)	55	33.0
cucullatus (Hooded Merganser)b	5	6.5		50	31.0
serrator serrator (Red-breasted Merganser)	6	5.66	(0.79)	72	32.0
serrator serrator (Red-breasted Merganser)b	10	6.1		66	29.0
merganser merganser (Goosander)a	4	14.9	(2.05)	69	32.0
Oxyurini					
Oxyura					
leucocephala (White-headed Duck)	3	19.33	(5.03)	96	25.0
leucocephala (White-headed Duck)a	5	20.9	(3.79)	92	22.0
jamaicensis (North American Ruddy Duck)	11	20.11	(7.35)	73	24.5
jamaicensis (North American Ruddy Duck)a	9	20.3	(1.91)	74	21.0
vittata (Argentine Ruddy Duck)a	3	22.7	(1.69)	87	21.0
maccoa (African Maccoa Duck)	3	24.23	(1.96)	96	26.0
Biziura					
lobata (Musk Duck)	1	21.79		128	?
Heteronetta					
atricapilla (Black-headed Duck)	2	18.73	(4.14)	60	21.0
Phoenicopteridae					
Phoenicoparrus					
andinus (Andean Flamingo)	1	21.16		?	27.0 – 31.0

? = Data unavailable

References :- a = Hoyt *et al.* (1979) ; b = Morgan, unpudlished data quoted in Hoyt *et al.* (1979) ; c = Ar & Rahn (1978) ; d = Snyder *et al.* (1982)

Table 2. Estimated artificial incubator humidities for 36 species of Anatidae.

SPECIES	No.	G_{H_2O} (mg/day/torr)	$\dot{M}_{H_2O}$+ (mg/day)	Incubator Humidity P(torr)	Incubator Humidity RH%
Dendrocygna bicolor	27	15.0	266	29.3	62.2
D. arborea	15	14.3	240	30.3	64.4
D. viduata	9	8.4	180	25.6	54.4
D. autumnalis	10	11.6	214	28.6	60.8
Anser fabilis	11	26.5	701	20.6	43.8
A. erythropus	10	21.9	545	22.2	47.2
A. canagicus	13	25.4	670	20.7	44.0
A. domesticus	11	27.7	816	17.6	37.4
Branta leucopsis	22	19.7	597	16.8	35.7
Tadorna variegata	8	13.0	404	16.0	34.0
T. tadorna	8	13.1	347	20.6	43.8
Neochen jubatus	8	10.7	280	20.9	44.4
Anas v. versicolor	11	5.7	181	15.3	32.5
A. v. puna	11	7.6	239	15.6	33.1
A. gibberifrons gracilis	17	7.7	195	21.7	46.1
A. p. platyrhynchos	15	13.7	259	28.2	59.9
A. p. diazi	10	12.0	290	22.9	48.7
A. boscas	11	14.5	394	19.9	42.3
Somateria m. mollissima	9	21.2	562	20.6	43.8
Netta ruffina	8	10.3	280	19.9	42.3
Aythya novoe-seelandioe	8	12.4	296	23.2	49.3
A. fuligula	8	9.1	320	11.9	25.3
A. affinis	8	8.0	291	10.7	22.7
Calonetta leucophrys	30	5.8	160	19.5	41.4
Aix galericulata	14	5.3	189	11.4	24.2
A. sponsa	26	6.3	196	16.0	34.0
Sarkidornis m. melanotos	10	8.3	293	11.8	25.1
Cairina moschata	13	11.8	278	23.5	49.9
C. scutulata	13	20.1	280	33.1	70.3
Bucephala islandica	15	9.7	290	17.2	36.5
B. c. clangula	14	10.6	253	23.2	49.3
Mergus albellus	14	9.1	202	24.9	52.9
M. cucullatus	15	7.4	244	14.1	30.0
M. s. serrator	16	5.9	298	-3.4	-
Oxyura leucocephala	8	20.3	524	21.3	45.3
O. j. jamaicensis	20	20.2	407	26.9	57.1

+ $\dot{M}_{H_2O}$ required for the egg to lose 12% of its fresh egg weight (equation 4)

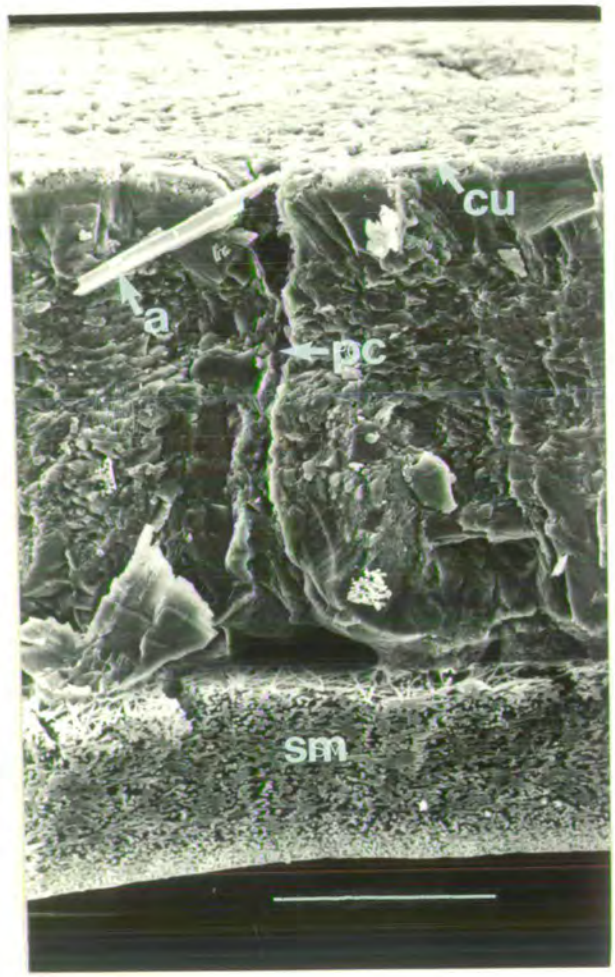
Table 3 : Measured humidity of Anatidae nests

SPECIES	NO.	Ref.	Method	P(nest)
Cygnus atratus	3	1	A	22.4
C. c. cygnus	1	1	A	32.9
Anser coerulcens	1	3	B	24.2
Anser anser	1	3	B	22.3
Branta leucopsis	3	1	A	18.2
Alopechen aegyptiacus	1	3	B	19.2
Anas p. platyrhynchos	1	2	B	26.7
	1	3	B	17.4
Somateria m. mollissima	1	2	B	23.6
Aythya novoe-seelandioe	1	2	B	15.3
Oxyura leucocephala	2	2	B	21.5
O. vittata	1	2	B	26.0

Methods:- A = electronic measurement ; B = egg hygrometry.
References:- 1 = Howey(1982) ; 2 = French (unpublished observations); 3 = Rahn et al.(1977)

Fig 1. Radial fracture of a Pink-eared Duck (Malacorhynchus membranaceus) eggshell showing respiratory pore; pc = pore canal, cu = cuticle, sm = shell membrane, a = artifact, bar = 100um. Electron micrograph taken on the JOEL 35C SEM.

Figure 1



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OXYGEN FLUX ACROSS THE INTEGUMENT OF THE AVIAN EGG DURING INCUBATION

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1. The oxygen flux across the integument of eggs of domestic fowl, domestic duck, mallard, moorhen, pheasant and turkey shows a ten-fold increase during the first week of incubation.

2. Suggestions are made as to how this is brought about. An assessment is also made of the contribution of the various components to the overall " resistance " of the integument.

INTRODUCTION

The avian embryo develops within a rigid calcitic shell. Firmly secured to the inner surface of the shell is the outer shell membrane and attached to this, except where they separate to form the air space at the blunt pole of the egg, is the inner shell membrane. This compound membrane has a fibrous net-like structure which is porous (Hays and Sumbardo, 1927). The pore system in the shells used in this study is the classical funnel-shaped pore which traverses the shell radially; the wider outer orifice being occluded in the pheasant, plugged by the organic cuticle which covers the external surface of the eggs of the domestic fowl and turkey or merely bridged by cuticle as in the domestic duck (see Fig. 1 and Board *et al.*, 1976). This cuticle-shell-membrane barrier or integument serves numerous functions: it provides mechanical protection for the embryo, it is the first line of defence against microbial infection and it is the avenue of exchange, via the pores, of respiratory gases and loss of water by the egg.

The oxygen consumption of the avian embryo increases during incubation (Romanoff, 1967). It has been found, however, that the oxygen flux across the shells of White Leghorn chicken eggs from which the inner shell membrane has been removed does not change during incubation and is initially sufficient to meet the high oxygen demands of the embryo in the later stages of incubation (Wangensteen *et al.*, 1970/71). These workers concluded that the porosity of the egg shell *per se* determines its permeability, that the optimal porosity has been fixed by evolution and that this is set at the time of laying.

In contrast, other workers have found that when the compound membrane is intact the oxygen permeability of the integument in newly laid eggs is not sufficient to permit the increase in oxygen consumption which occurs in later development (Kutchai and Steen, 1971). A sudden increase in the oxygen permeability, to

values which would allow this higher oxygen consumption, was observed between the second and fifth days of incubation in the eggs of the domestic fowl. Further, this sudden increase occurred only in fertilised eggs where measurements by these authors indicated that it may be connected with a progressive drying of the compound membrane during incubation. Such conclusions are in agreement with the

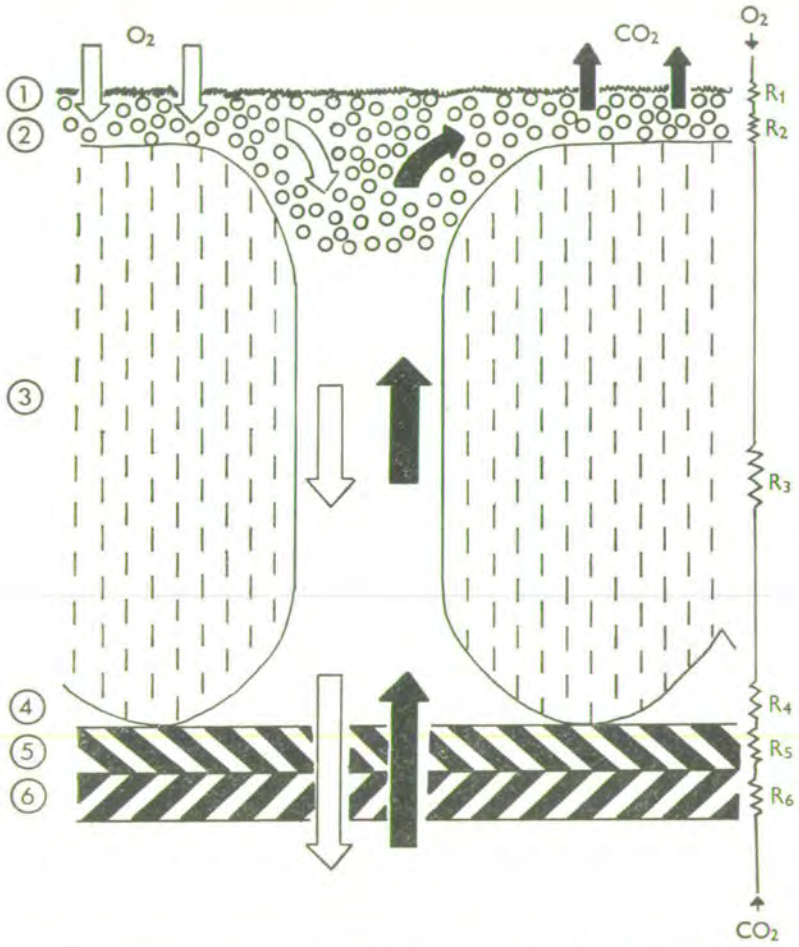

FIG. 1.—Schematic representation of an avian egg shell (domestic fowl type) showing the main structural features. A layer of preening oil (1) is shown deposited on the cuticle (2). The column layer (3), cone layer (4), inner (5) and outer (6) shell membranes are also depicted. A resistance network for the diffusion of gases across the integument has been constructed on the right of the figure.

observations and hypotheses of Romijn (1950) and also with the work of Romanoff (1943) who found an increase in the filtration coefficient of the shell with incubation.

That the permeability of avian egg shells increases with incubation has been challenged by Wangensteen *et al.* (1970/71) on the basis of their oxygen flux determinations and by Rahn *et al.* (1972) on the grounds that under conditions of constant humidity and temperature the loss of water from fertilised eggs remains remarkably constant throughout incubation.

The present work was undertaken to investigate this apparent discord and to assess the contribution of the various components to the total resistance of the integument to oxygen diffusion. Further, a preliminary examination of changes which may affect the integument has been made during the early stages of incubation.

MATERIALS AND METHODS

Sources of eggs

Eggs of the domestic fowl (*Gallus domesticus*), pheasant (*Phasianus colchicus*), and turkey (*Meleagris gallopavo*) were obtained from commercial producers; additional pheasant eggs were provided by the Game Conservancy, Fordingbridge; mallard (*Anas platyrhynchos*) and moorhen (*Gallinula chloropus*) were from the Wild-fowl Trust, Slimbridge; domestic duck eggs were from a small free range flock.

Measurement of oxygen flux

Fertile eggs at various stages of incubation were cut around their shoulder. The narrow pole was removed, the inner shell membrane wiped clean of adhering albumen with a tissue and the oxygen diffusion across the cuticle-shell-compound membrane barrier immediately measured.

Measurements were made using apparatus similar to Kutchai and Steen (1971) and the results expressed as described by Wangensteen *et al.* (1970/71). The surface area of the shells used was determined by breaking them up into relatively flat pieces and passing these through an automatic surface area recorder (Hayashi Denko Co. Ltd, Tokyo, Japan).

Measurement of water content of egg albumen

The percentage water content by weight was measured by drying a known weight of albumen over a desiccant.

Photography

Samples of compound membrane were removed dry from the inside of egg shells and placed under a cover slip on a glass slide. The membranes were photographed (Panatomic X, Kodak, London) before, and 10 min after, contact with water in areas that were thin due to tearing of the fibres during removal from the shell.

RESULTS

The oxygen flux across the narrow pole of fertile eggs of domestic fowl, pheasant, turkey, domestic duck, mallard and moorhen are shown plotted against days of incubation in Fig. 2*a-d*. All these species showed an increase (from around 1×10^{-7} to 1×10^{-6} cm^3 STP s^{-1} cm^{-2} $mmHg^{-1}$) in oxygen permeability within the first 8 d of

incubation. No increase in oxygen diffusion was recorded in infertile eggs but it was, however, observed in eggs in which there appeared to be a normal accumulation of sub-embryonic fluid without the development of an embryo or vascular system.

In fertile eggs of the domestic fowl (days 4 to 6), pheasant (days 5 to 7) and turkey (days 6 to 8) there was a sudden increase in the oxygen permeability to a fairly uniform level, while in the domestic duck and mallard there was a more gradual increase to a less uniform level. In all these species the oxygen flux increased to levels which could be obtained experimentally in several ways, for example, removing the wet inner shell membrane of infertile eggs of unincubated fertile eggs. It could also be attained by taking low O_2-flux unincubated fertile or infertile

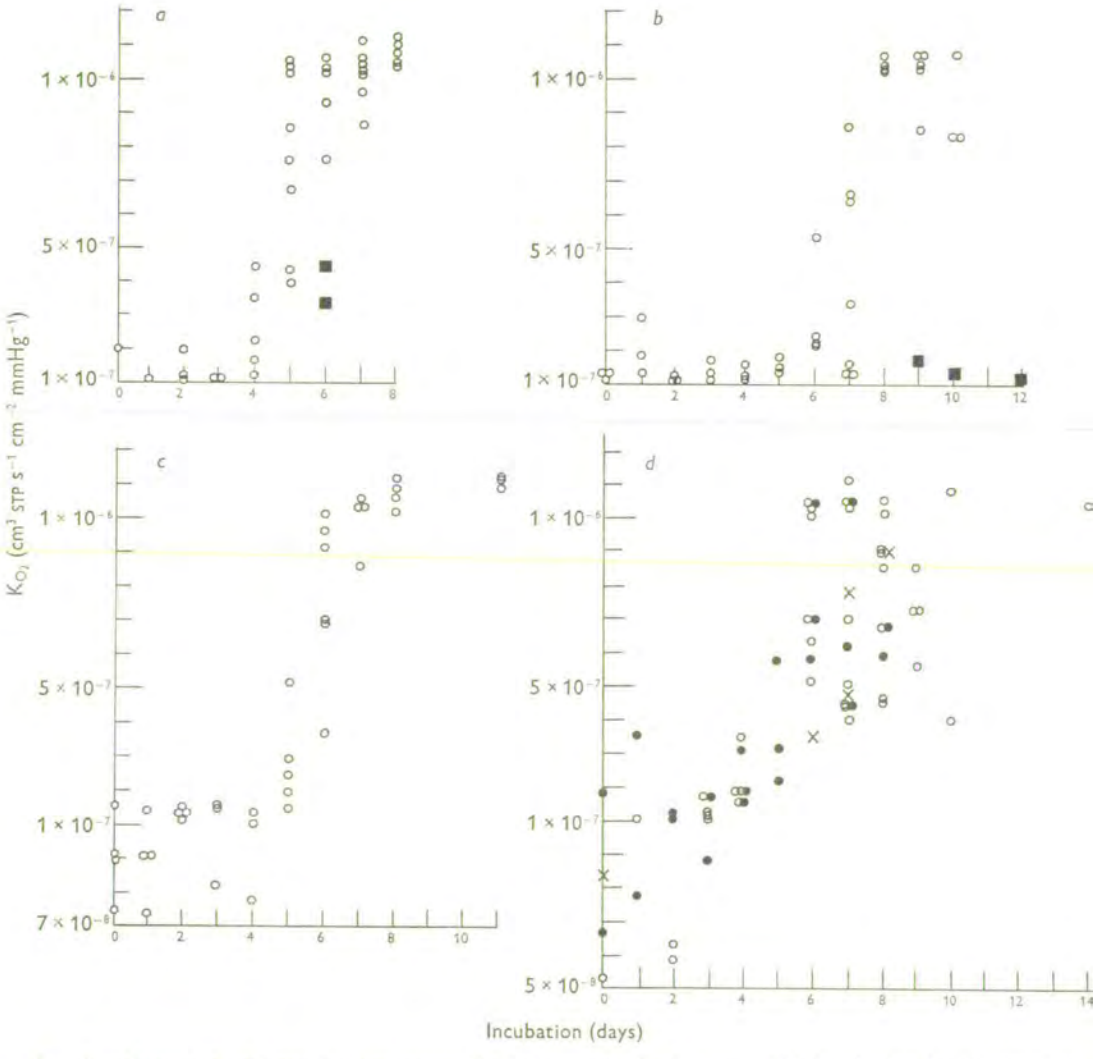

FIG. 2.—Changes in the oxygen flux across the integument of avian eggs with incubation for (*a*) domestic fowl (■ symbols represent infertile eggs), (*b*) turkey, (*c*) pheasant and (*d*) mallard (○), domestic duck (●) and moorhen (×).

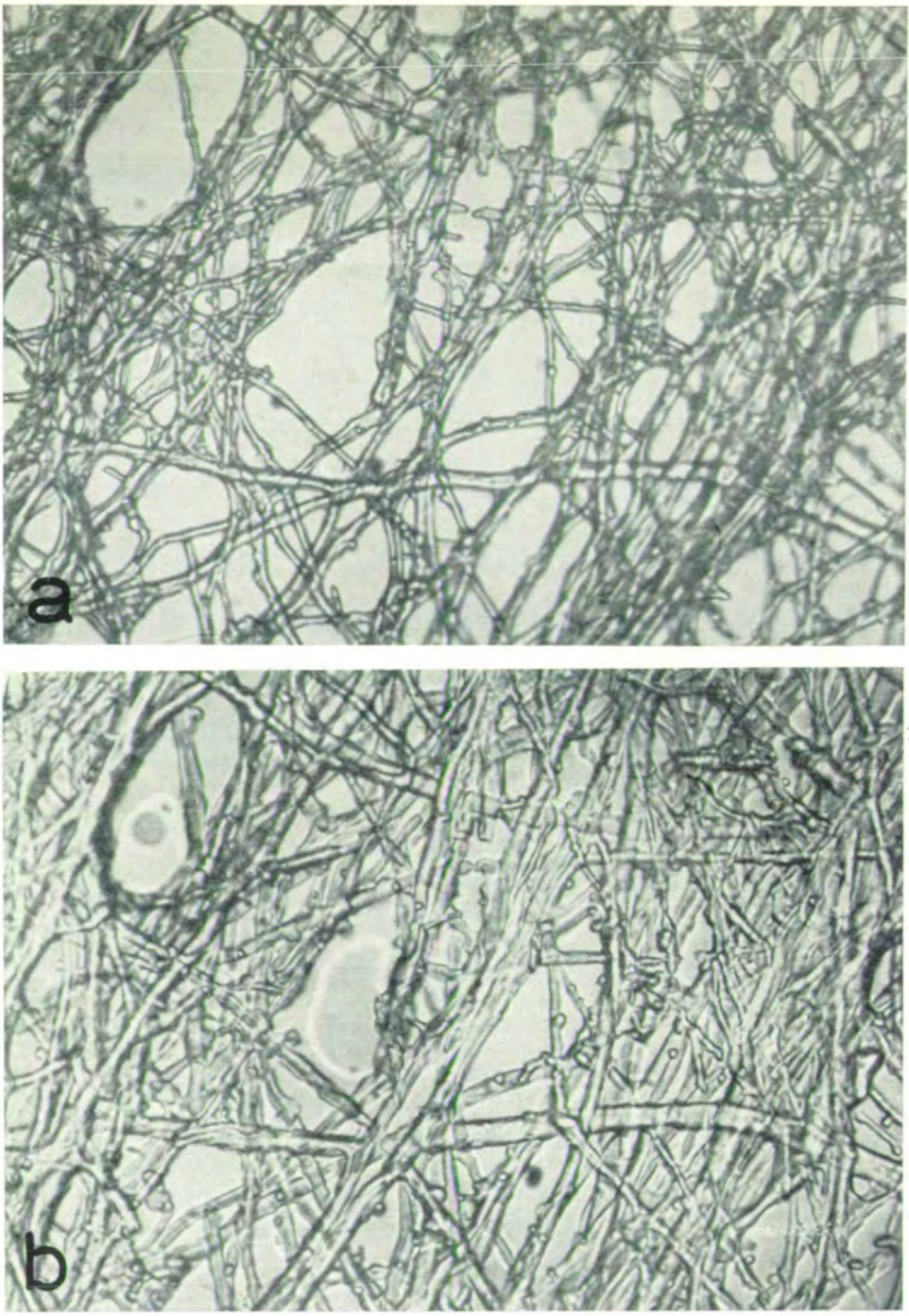

PLATE-FIG. 1—(*a*) View at the edge of a dry piece of inner shell membrane which shows, owing to tearing on removal from the egg, one layer of fibres; (*b*) was taken 10 min after water had been introduced under the coverslip. (× 1000.)

eggs and thoroughly drying the excised narrow pole over a desiccant. Further, if these dried, high-O_2 shells or high-O_2 day 8 fertile shells were then filled with water for a short time, emptied and then wiped inside with a tissue, the low O_2-flux was re-established. Under the light microscope the fibres in a dry compound membrane are seen to expand in width when they are in contact with water (Plate-Fig. 1). Such observations suggest that the degree of hydration of the inner shell membrane may be important in the determination of the resistance to oxygen diffusion across the cuticle-shell-compound membrane barrier. Measurements of the water content of the egg white which is in contact with this membrane throughout the early part of incubation were therefore made.

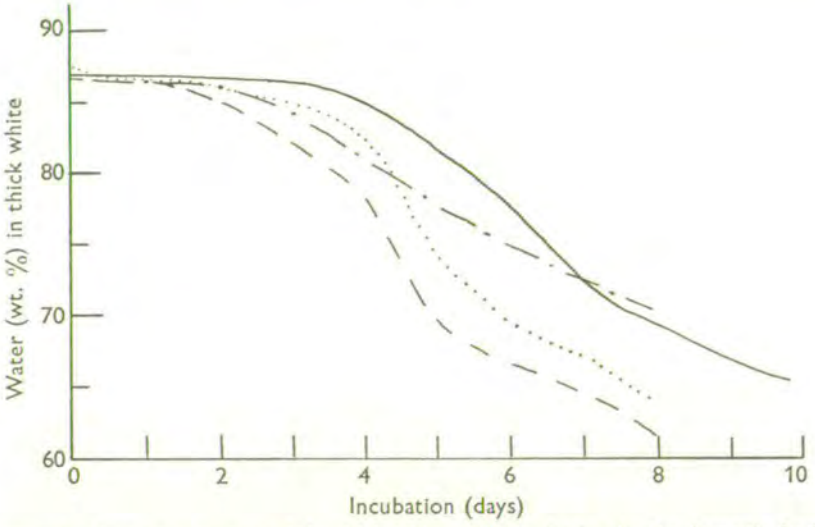

FIG. 3.—Changes in the water content of the thick white with incubation in the domestic fowl (— — —), pheasant (. . . . .), turkey (———) and domestic duck/mallard (— · — · —).

The thin white, which is apposed initially to the inner shell membrane, disappears early in incubation without a significant change in its water content. For example, in the domestic fowl the thin white contains initially about 86% by weight of water and disappears without a change in this level by the end of day 5. The thick white, however, which is now apposed to the membrane, becomes successively concentrated in fertile eggs (Fig. 3). No significant change occurred in the water content of the thick white in infertile eggs but eggs in which there was a normal accumulation of sub-embryonic fluid but little embryo or vascular development showed changes characteristic of fertile eggs. It is noteworthy that the sudden decrease to 70% by weight of water in the thick white is associated temporally with the sudden increase in the oxygen flux across the shells of fertile eggs of domestic fowl, pheasant and turkey. The decrease in the water content of the thick white in the domestic duck and mallard is more gradual. Such an observation suggests that an increase in the osmotic pressure of the thick white may result in water being drawn from the compound membrane thus leading to an increase in the oxygen permeability. A slight increase in the osmotic pressure

(measured by the depression of freezing point) was found, for example in the domestic fowl (from 0·26 Osm on day 0 to 0·50 Osm on day 6) and turkey (from 0·22 Osm on day 0 to 0·35 Osm on day 9). However, neither filling the narrow pole of an unincubated egg (which had just been wiped clean inside) with a concentrated sucrose solution nor thick white from advanced eggs for periods of up to 20 h increased the oxygen permeability by more than 10% compared with the 1000% increase occurring during incubation.

Finally, measurements of oxygen diffusion across the narrow pole of four consecutive infertile eggs laid by a Rhode Island Red hen were taken in order to determine the contribution of each component to the overall resistance to diffusion of the cuticle-shell-compound membrane barrier. The results are presented in Table 1, the figures in brackets indicate the percentage increase in oxygen flux as a result of a particular treatment over the previous one.

TABLE 1

The effect of various treatments on the K_{O_2} across the integument of domestic fowl eggs*

	Treatment 1 Sharp pole removed from egg and inner shell membrane wiped clean of albumen	Treatment 2 Sharp pole from 1 rinsed in cold water and then thoroughly dried over desiccant at 40 °C	Treatment 3 Shell from 2 boiled for 10 min in 5% NaOH, rinsed in tap water to remove the softened cuticle and replaced in NaOH for further 10 min. Examined to ensure complete removal of cuticular plugs and thoroughly dried
Egg no. 1	$1{\cdot}37 \times 10^{-7}$	$2{\cdot}39 \times 10^{-6}$ (1644%)	$2{\cdot}55 \times 10^{-6}$ (6·7%)
2	$1{\cdot}37 \times 10^{-7}$	$2{\cdot}57 \times 10^{-6}$ (1776%)	$2{\cdot}66 \times 10^{-6}$ (3·5%)
3	$1{\cdot}37 \times 10^{-7}$	$2{\cdot}44 \times 10^{-6}$ (1608%)	$2{\cdot}50 \times 10^{-6}$ (2·5%)
4	$1{\cdot}37 \times 10^{-7}$	$2{\cdot}18 \times 10^{-6}$ (1491%)	$2{\cdot}35 \times 10^{-6}$ (7·8%)

* K_{O_2} is measured as cm^3 STP s^{-1} cm^{-2} $mmHg^{-1}$.

These figures clearly show that the membranes when wet do not permit the full oxygen flux potential of the shell to be realised. This " brake " function appears to be confined to the inner shell membrane (R_6 in Fig. 1) for when this is peeled off the outer shell membrane (R_5) appears dry and an oxygen flux equivalent to late incubation can be measured. Further, eggs of some other precocial birds had a similar oxygen flux when thus treated (Table 2).

The cuticle of the domestic fowl egg has a negligible resistance (R_2) to oxygen flux across the integument in eggs taken fresh from laying birds and incubated in the incubator. However, naturally incubated eggs have been shown to become smeared with oil from the parents preening gland (Board *et al.*, 1976). Samples of this oil were removed from the preening gland of Aylesbury ducks with a cotton wool swab and dissolved in a small quantity of petroleum ether. When this solution was applied to the cuticle of a domestic fowl egg shell the oxygen flux was reduced. Cursory examinations suggested that the resistance to oxygen diffusion of the preening oil layer (R_1) may change with temperature presumably as a result of some phase change within the oil (Table 3) a situation analogous to the insect cuticle (Beament, 1961).

TABLE 2

O_2 flux across the cuticle-shell-outer shell membrane barrier of the Muscovy duck (Cairina moschata), *moorhen, guinea fowl and pheasant*

Species	Egg no.	Area of egg[1]	K_{O_2} ($\times 10^{-6}$)*
Muscovy duck	1	B	1·82
		S	1·83
	2	B	1·17
		S	1·46
	3	B	1·70
		S	1·75
	4	B	1·50
		S	1·67
Moorhen	1	B	1·35
		S	0·98
	2	B	1·56
		S	Cracked
	3	B	1·60
		S	1·11
Guinea fowl	1	B	2·44
		S	1·97
	2	B	2·31
		S	Cracked
	3	B	2·42
		S	1·62
	4	B	2·34
		S	Cracked
Pheasant	1	S	1·98
	2	S	1·70
	3	S	1·64
	4	S	1·38
	5	S	1·73
	6	S	1·70

[1] B = wide pole; S = narrow pole.

* K_{O_2} is measured as cm^3 STP s^{-1} cm^{-2} $mmHg^{-1}$.

DISCUSSION

The oxygen flux across the cuticle-shell-compound membrane barrier in freshly laid eggs has been shown to be insufficient to meet the oxygen demands made by the embryo in the later stages of incubation, a finding in agreement with the work of Kutchai and Steen (1971). An increase in the oxygen permeability of this barrier (from around 1×10^{-7} to 1×10^{-6} cm^3 STP s^{-1} cm^{-2} $mmHg^{-1}$) occurs within the first 8 d of incubation in fertile eggs of the domestic fowl, pheasant, turkey, domestic duck, mallard and moorhen. Such an increase can only be brought about experimentally in freshly laid eggs if the barrier is thoroughly dried or if the wet inner shell membrane is removed.

In the course of incubation the increase in oxygen flux across the shell takes place before the vascular system is well established and also occurs in eggs showing a normal accumulation of sub-embryonic fluid but with little embryo or vascular development. Therefore, although the vascular system may play a rôle in keeping

the membranes dry the actual drying appears to be caused by other mechanisms. In the domestic fowl the first 2 d of incubation are characterised by an enlargement of the blastoderm around the upper yolk. Sub-embryonic fluid accumulates between the embryo and yolk after day 2. According to New (1956) the fluid is formed by the blastoderm which actively absorbs water from the albumen and secretes it from its endoderm surface. The accumulation of this fluid inflates the yolk sac and by the fourth day or so the blastoderm is separated from the inner shell membrane by only a thin layer of albumen. At this time the thin white has largely disappeared and the thick white subsequently becomes increasingly concentrated. However, the present work has shown that a rise in osmotic pressure in the albumen of fertile

TABLE 3

Effect of temperature and preening oil on the permeability of the shell of the domestic fowl to oxygen

Sample	Temperature (°C)	K_{O_2}*
Narrow pole of domestic fowl egg minus inner shell membrane	22	$2{\cdot}35 \times 10^{-6}$
Above specimen smeared with preening oil solution	22	$6{\cdot}88 \times 10^{-7}$
	40	$9{\cdot}65 \times 10^{-7}$
Preening oil removed by washing in petroleum ether	22	$2{\cdot}35 \times 10^{-6}$

* K_{O_2} is measured as cm^3 STP s^{-1} cm^{-2} $mmHg^{-1}$.

eggs is not in itself sufficient to alter the oxygen permeability of the shell. In this respect, figures calculated from the work of Romanoff (1967) on eggs of the domestic fowl are worthy of examination and are presented in Table 4. These show that during the first 6 d of incubation there is a loss of water from the egg white and a gain of water by the sub-embryonic fluid. Moreover, the figures indicate that there is probably a direct transfer between the two compartments. Loss of sodium from the egg albumen is associated with an equivalent gain of sodium by the sub-embryonic fluid which suggests the involvement of a sodium pump mechanism. If such a pump is present within the blastoderm this may account for the importance of turning in domestic fowl eggs between days 4 and 7 of incubation (New, 1957) which would ensure that the blastoderm comes into contact with virtually all the inner surface of the inner shell membrane within each 24-h period.

Although it has been shown that water causes the membrane fibres to expand, thus leading to a decrease in the area available for gaseous diffusion (Plate-Fig. 1), Wangensteen *et al.* (1970/71) maintain that this effect would not be sufficient to decrease significantly the permeability of the barrier. The proposition that there is a layer of water in the pores of the compound membrane which becomes thinner and thinner as the membrane dries and finally abruptly ruptures in some places to create air-filled channels (Kutchai and Steen, 1971) is perhaps more appropriate therefore to explain the sudden increase in oxygen flux which occurs with incubation.

That the permeability of avian egg shells increases with incubation has been challenged by Rahn *et al.* (1974) on the grounds that under conditions of constant humidity and temperature the daily weight loss from fertile eggs remains remarkably constant throughout incubation. It is suggested in the present study that the

oxygen flux increases as a result of drying of the inner shell membrane and the above authors (Paganelli *et al.*, 1973) have already found that the membranes offer a negligible resistance to water movement across the domestic fowl egg shell. A dehydration of the compound membrane during incubation also has implications in other directions, for example, the consequent decrease in the water activity could account for the difficulty in establishing rot in fertile eggs referred to by Board and Fuller (1974).

The O_2 flux of the cuticle-shell-outer shell membrane as measured by

TABLE 4

Changes in water and sodium content of the egg albumen and sub-embryonic fluid of the incubated domestic fowl egg (calculated from Romanoff, 1967)

Day of incubation	Amount of water (g) in egg albumen	Change	Amount of water (g) in sub-embryonic fluid	Change	Sodium (mg) in total egg albumen	Change	Sodium (mg) in sub-embryonic fluid	Change
0	29·90		...		40·58		...	
		−1·00		...		−1·68		...
1	28·90		...		38·90		...	
		−1·49		+0·62		−1·68		+1·5
2	27·41		0·62		37·27		1·5	
		−3·12		+2·27		−1·71		+4·8
3	24·29		2·89		35·51		6·3	
		−3·95		+3·33		−5·29		+4·8
4	20·34		6·22		30·22		11·1	
		−4·83		+3·78		−5·28		+4·8
5	15·51		10·00		24·94		15·9	
		−4·69		+2·93		−5·28		+4·9
6	10·82		12·93		19·66		20·8	
Total change		−19·08		+12·93		−20·92		+20·80

Note—A further 2 g of water will have been lost by evaporation through the pores and a total gain of 1·3 g has been made by the yolk sac and contents, allantoic membrane and allantoic fluid, amniotic membrane and amniotic fluid, whole embryo and extra-embryonic blood.

Wangensteen *et al.* (1970/71) is fixed at the time the egg is laid for presumably the outer shell membrane dehydrates quickly by water loss through the pores in the egg shell. It represents the level attained in fertile eggs when the high resistance of the inner shell membrane is removed during incubation. The cuticle and outer shell membrane offer negligible resistance to oxygen diffusion. Hence, the resistance of the true shell (R_3) determines the potential O_2 flux of the avian egg shell and it is the porosity of the true shell which must be modified by evolutionary pressures, as proposed by Wangensteen *et al.* (1970/71). The manner in which changes in egg shell porosity may be brought about has been discussed previously (Tullett, 1975). It has been repeatedly stressed that the avian egg shell performs so many functions that its microstructure in different species must reflect some form of evolutionary compromise depending on the egg/nest/environment (Board and Fuller, 1974; Tullett *et al.*, 1975; Board *et al.*, 1976). Rahn (1972) has implicated that where such a compromise results in a shell having a low oxygen permeability the bird will give rise to altricial rather than precocial young.

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REFERENCES

Beament, J. W. L. (1961). The water relations of insect cuticle. *Biol. Rev.*, **36**: 281-320.

Board, R. G. and Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biol. Rev.*, **49**: 15-49.

Board, R. G., Tullett, S. G. and Perrott, H. R. (1976). A classification of pore systems in avian eggshells. Submitted to *J. Zool.*

Hays, F. A. and Sumbardo, A. H. (1927). Physical characters of eggs in relation to hatchability. *Poult. Sci.*, **6**: 196-200.

Kutchai, H. and Steen, J. B. (1971). Permeability of the shell and shell membranes of hen's eggs during development. *Resp. Physiol.*, **11**: 265-278.

New, D. A. T. (1956). The formation of sub-blastodermic fluid in hen's eggs. *J. Embryol. exp. Morph.*, **4**: 221-227.

New, D. A. T. (1957). A critical period for the turning of hen's eggs. *J. Embryol. exp. Morph.*, **5**: 293-299.

Paganelli, C. V., Ar, A. and Rahn, H. (1973). Relative water vapour permeabilities of the shell and shell membranes of the hen's egg. *Physiologist*, **16**: 415.

Rahn, H. (1972). Egg shell porosity and gas conductance differences in a precocial chicken and an altricial bird (double crested cormorant (*Phalacrocorax auritus*)). *The Bulletin, Mt Desert Island Biol. Lab., Maine*, **12**: 79-80.

Rahn, H., Paganelli, C. V. and Ar, A. (1974). The avian egg: air-cell gas tension, metabolism and incubation time. *Resp. Physiol.*, **22**: 297-309.

Romanoff, A. L. (1943). Study of various factors affecting permeability of bird's eggshell. *Food Res.*, **8**: 212-223.

Romanoff, A. L. (1967). *Biochemistry of the Avian Embryo.* New York, John Wiley and Sons.

Romijn, C. (1950). Foetal respiration in the hen. Gas diffusion through the eggshell. *Poult. Sci.*, **29**: 42-51.

Tullett, S. G. (1975). Regulation of avian eggshell porosity. *J. Zool.*, **177**: 339-348.

Tullett, S. G., Lutz, P. L. and Board, R. G. (1975). The fine structure of the pores in the shell of the hen's egg. *Br. Poult. Sci.*, **16**: 93-95.

Wangensteen, O. D., Wilson, D. and Rahn, H. (1970/71). Diffusion of gases across the shell of the hen's egg. *Resp. Physiol.*, **11**: 16-30.

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CHANGES IN STRUCTURE OF THE LIMITING MEMBRANE AND IN OXYGEN PERMEABILITY OF THE CHICKEN EGG INTEGUMENT DURING INCUBATION

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Running headline: PERMEABILITY OF SHELL MEMBRANES

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1. Oxygen permeability (K_{O_2}) of the shell and shell membranes of fertile and infertile chicken eggs were measured throughout 14 days incubation with turning at 37.5°C and a relative humidity of 60%. The K_{O_2} of the shell and membranes of infertile eggs was around 1.0×10^{-7} cm^3 O_2STP. sec^{-1} cm^{-2}. $Torr^{-1}$ throughout incubation. With fertile eggs, from which there was a linear loss of water during incubation, the K_{O_2} of the shell and shell membranes was about 1.0×10^{-7} cm^3 O_2STP.sec^{-1} cm^{-2}. $Torr^{-1}$ for the first 4 days of incubation. Thereafter the majority of shells and membranes had a K_{O_2} of about 1.0×10^{-6} cm^3 O_2STP.sec^{-1} cm^{-2}. $Torr^{-1}$. (Torr ≈ 133.322 Pa).

2. A diminution of the Na^+ and K^+ content of the shell membranes of fertile eggs was not associated with changes in the dimensions of the glycoprotein mantle on the cores of the individual fibres of the membranes. There was, however, a progressive deterioration in the limiting membrane of fertile but not infertile eggs.

3. It was concluded that changes in the O_2 resistance of the integument of fertile eggs were not a product of change in either of the shell membranes but of damage caused to the limiting membrane by the chorioallantois.

INTRODUCTION

Several studies have shown that an 10-fold increase in the oxygen permeability of the integument (the porous calcitic shell lined on its outer surface with cuticle and on its inner surface with fibrous membranes) occurs 22% of the way through incubation of the fertile eggs of several species of birds (Kutchai and Steen, 1971; Lomholt 1976; Tullett and Board, 1976; Kayar et al., 1981), providing that the integument does not dry out before or during determinations (Wangensteen et al., 1970/1971). Indeed attempts to interpret this observation have invoked discussion about the role of water, either in or between the fibres of the shell membranes, on the diffusion of O_2 through the interstitial spaces. Thus with the latest study (Kayar et al., 1981), the hypothesis was advanced that the increased permeability of the inner membrane, which these authors found to account for 88% of the initial resistance of the integument to oxygen flux, was due to evaporation causing a layer of water in this membrane to be reduced in thickness from about 63 - 0.6 μm.

Before any hypothesis that attempts to explain changes in the oxygen permeability of shell membranes in terms of the latters' content of water is used to plan further experiments, it is pertinent to consider both the fine structure and chemical composition of the shell membranes. Although the literature quoted above conveys the impression that the avian shell is lined with only two membranes,Bellairs and Boyde (1969) found in studies of fine structure with the electron microscope that the inner surface of the main shell membrane is covered with electron dense material, which they called the limiting membrane. It is notable also that a narrow band of electron dense material separating the main shell membrane and the chorioallantois has been observed repeatedly (Skalinsky and Kondalenko, 1963; Leeson and Lesson, 1963; Froix et al., 1977). Thus in practice there are good reasons for considering that there are three resistances, the inner and outer shell membranes and

the limiting membrane, to the inward diffusion of oxygen that has passed through the pores in the calcitic shell. Moreover the diameters of the fibres of the first two membranes vary appreciably, those in the outer shell membrane being upwards of 3-6 μm and those of the inner being as little as 0.4 μm. Furthermore the fibres have a composite structure, a central core of a fibrous protein rich in desmosine and isodesmosine (Harris et al., 1980) but resistant to elastase (Leach and Rucker, 1978; Starcher and King, 1980; Crombie et al., 1981; Leach et al., 1981) is surrounded by a glycoprotein mantle which probably contains glucose, galactose, mannose, xylulose, glucosamine, galactosamine and sialic acid (Baker and Balch, 1962; Cooke and Balch, 1970; Wedral et al., 1974). The present study was undertaken with the objectives of studying the fine structure of the shell and limiting membranes of the incubating eggs of the domestic chicken and attempting to associate the diminution in the resistance of the egg's integument to oxygen flow with changes in fine structure.

MATERIALS AND METHODS

Eggs

Eggs of the domestic hen were obtained from local farm hens housed in batteries (infertile eggs) or from a local commercial hatchery (fertile eggs). The eggs were used immediately or incubated in a small incubator (Brinsea Products Ltd., West Brinsea Farm, Congresbury, Avon, U.K.) maintained at 37.5^{o}C and 60% RH. The eggs were turned automatically every hour.

Glassware

All glassware was thoroughly cleaned by soaking in concentrated nitric acid overnight and washing in deionised glass-distilled water.

Measurement of K_{O_2}

The oxygen flux across the shell and shell membranes of both fertile and infertile hen eggs was measured using the apparatus described by Kutchai and Steen (1971). Eggs at various stages of incubation were cut around their shoulder and the narrow pole wiped clean of adhering albumen with a tissue. The shell was immediately placed in the apparatus and sealed around the shoulder with "Xantoprene" polymer (Bayer Ltd.). The enclosed space beneath the shell was flushed with humidified N_2 until the O_2 concentration as measured by the electrode of a WPA O_2 meter (Walden Precision Apparatus Ltd., Saffron Walden, Essex, U.K.) was zero. The gas inlet and outlets were closed and the time taken to reach half the maximum O_2 concentration recorded on paper charts.

The surface area of the eggshells was determined by breaking them up into small pieces and passing these through an automatic surface area recorder (Lambda Instruments Corporation, Lincoln, Nebraska, U.S.A.). The results were expressed as described by Wangensteen et al. (1970/1971).

Ionic content of shell membranes

Eggs were cracked open and their contents of white and yolk removed. The shell membranes were washed (x10) with deionised glass-distilled water, removed from the shell, and dried to a constant weight in a hot-air drying oven at 80°C. The dried membranes were ground to a fine powder using a glass pestle and mortar and ashed in a muffle furnace overnight at 450°C or until a whitish-gray ash remained. If difficulty was experienced in obtaining a carbon-free ash the ash was cooled, moistened with deionised water, evaporated to dryness at 80°C and reheated to 450°C. When all the organic matter was destroyed the ash was cooled and a solution of the ash prepared as for plant material (Anon., 1973).

Sodium and potassium were analysed using the flame photometer (Corning Ltd., Halstead, Essex, U.K.) and calcium and magnesium analysed by atomic absorption using the atomic absorption spectrophotometer SP1600 (Pye-Unicam Ltd.). In both cases standard solutions of the elements (Fisons Ltd.) were used as detailed in the ADAS Technical Bulletin No. 27 (Anon., 1973).

Electron Microscopy

Samples of shell membranes from the broad pole of eggs for scanning electron microscopy were fixed in 0.19M (v/v) gluteraldehyde in cacodylate buffer (pH 7.0) for 60 min at room temperature followed by dehydration in a series (8.60M-17.21M) acetone. The samples were dried to critical point using a critical point dryer (Polaron Equipment Ltd., Watford, London) and mounted on aluminium stubs. The specimens were coated with a thin layer of gold/palladium alloy under vacuum and examined using the Jeol 35C scanning electron microscope (Jeol (UK) Ltd., Colindale, London) at an accelerating voltage of 25Kv.

Samples of shell with membranes attached were prepared by freezing large sections of shell in liquid N_2 and then fracturing them into small pieces which were subsequently freeze dried (Edwards, Crawley, Sussex) and mounted on aluminium plates. The samples were then coated as described above and examined using the Jeol 35C scanning electron microscope.

Samples of shell membrane for transmission electron microscopy were fixed and stained in 0.19M (v/v) gluteraldehyde containing 1g/l (w/v) ruthenium red. After dehydration as described above the membranes were embedded in Taab E.M. resin (Taab Laboratories Equipment Ltd., Reading, Berkshire, England) and ultrathin sections, cut with a glass knife, collected onto uncoated copper grids. Sections were stained with a saturated solution of uranyl acetate in 14.86M (v/v) ethanol and Reynolds lead citrate for 15 min each and examined using the Jeol 100C transmission electron microscope at an accelerating voltage of 25 Kv.

RESULTS

Oxygen permeability

Oxygen permeability (K_{O_2}) through the integuments of 33 infertile hens eggs was about 1.0 x 10^{-7} $cm^3 O_2$ STP.sec^{-1} cm^{-2}.$Torr^{-1}$ throughout the 14 days of incubation at 37.5°C and 60% relative humidity (Fig. 1). The 24 fertile eggs examined up to the 4th day of incubation had K_{O_2} values similar to those of the infertile ones. After day 4, however, the resistance of the integuments of fertile eggs to oxygen diffusion diminished by about 10-fold and all the eggs examined gave K_{O_2} values of about 1.0 x 10^{-6} cm^3 O_2 STD.sec^{-1} cm^{-2}.$Torr^{-1}$ by the 10th day of incubation. These results, which are in accord with those of Kutchai and Steen (1971); Lomholt (1976); Tullett & Board (1976); Kayer et al. (1981), show clearly that some change(s) occur in the integument of fertile hens' eggs during the 4-6th day of incubation such that the potential for the inward flux of oxygen is increased. These changes were not reflected in the rate at which fertile eggs lost water by evaporation (Fig. 2).

Structure of shell membranes

It was evident in radial sections of the membranes taken from the shells of freshly laid eggs, both fertile and infertile, that there were three distinct layers (Fig. 3a), the outer two (the shell membranes sensu strictu) being distinguished one from the other by the diameter of their fibres, the larger ones being most numerous in the outer shell membrane. The limiting membrane, a narrow band of electron dense material, was a notable feature also (Fig. 3a). The core and mantle of individual fibres of the inner and outer shell membranes were evident with high powered magnification of ultra-thin sections stained with ruthenium red. Indeed a limited survey of the shell membranes of the eggs of several species of water fowl (Sparks & Board, unpublished

observations) and other bird species (Dr. L.J. Alberto, pers. comm.) indicates that composite fibres may be a common feature in avian eggs. It was noted during studies with a scanning electron microscope that differential fracture of the shell membrane fibres revealed several instances of 2 or more protein fibres contained within a common mantle (Fig. 3). Changes in the morphology of the shell membranes, especially in respect of the thickness of the mantle or the fibre, were not seen in incubated fertile or infertile eggs.

The limiting membrane appeared as an electron dense layer on the inner surface of the inner shell membranes examined by scanning electron microscopy (Fig. 4a). Its appearance in infertile eggs did not change during 14 days incubation with egg turning at 37.5°C and RH 60%. In marked contrast there was a progressive change in the microstructure of the limiting membrane of fertile eggs in which embryos developed (Figs. 4a,b and c). It needs to be stressed that all material studied with electron optics was taken from the broad end of the shell, the pointed end being used for determination of K_{O_2}. The first demonstrable changes, cracks in the limiting membrane (Fig. 4b), were found in fertile eggs incubated for 4 days. As incubation proceeded, the cracks became wider and, by the 18th day of incubation, only patches of the limiting membrane on the underlying fibrous inner shell membrane were evident (Fig. 4c).

Cation content of the shell membranes

Of the cations studied, the concentrations of Na^+ and K^+ in the shell membranes of fertile eggs diminished significantly during the first 3 days of incubation - but that of Na^+ increased thereafter - and that of Mg^{2+} between days 3-9. In contrast (Fig. 5), the concentrations of these cations in the membranes of infertile eggs decreased (Na^+) or increased (K^+, Mg^{2+}) slightly during the first 18 days of incubation. The marked increase in the concentrations of

K^+ and Na^+ by day 21 was attributed to the yolk making contact with the shell membranes of infertile eggs. The increase in the concentrations of Ca^{2+} and Mg^{2+} in the membranes of fertile eggs on day 21 probably reflects the carryover of tips of the cones from the inner surface of the shell. It is known (Board and Love, 1980) that the cones are relatively rich in Mg^{2+} and that their resistance to fracture is reduced as a consequence of the embryo absorbing Ca^{2+} from the shell (Bond, 1980).

DISCUSSION

The results presented above favour the conclusion that the decrease in the resistance of the integument of fertile hens' eggs to O_2 diffusion, which occurs about 22% of the time into incubation (Kutchai & Steen, 1971; Lomholt, 1976; Tullett and Board, 1976; Kayar et al., 1981), is caused, either wholly or in part, by changes in the limiting membrane. Before accepting evidence of change in the structure of such a delicate structure, we examined many limiting membranes from incubated fertile as well as infertile eggs which had been prepared in parallel in order to discount artefacts due to the preparation techniques. In every examination, cracks in the limiting membrane were noted only in fertile eggs which had been incubated for more than 4 days and in which normal embryo development occurred. Moreover there was an absolute correlation between a decrease in a membrane's resistance to oxygen diffusion and the occurrence of cracks. Additional support for the contention that there is an increase in O_2-diffusion across half-shells of incubated eggs (see Methods) comes from the studies of Temple & Metcalfe (1970) and Kayar et al. (1981). The former reported a progressive decrease in the resistance of the shell, shell membranes and chlorioallantois to oxygen diffusion in studies with whole eggs. Kayar et al. (1981) obtained good agreement between the mean K_{O_2} calculated from analysis of the difference between the Po_2 of the atmosphere and the Po_2 of the

air cell of fertile eggs incubated for 10-17 days and the K_{O_2} calculated from measurements with half shells and an oxygen electrode (i.e. the same method as that used by us). Thus two lines of evidence support the notion that changes in resistance to O_2 with incubation are the consequence of changes in the shell membranes that occurred during incubation and not during the preparative/recording stages of the experiments discussed here and elsewhere by Kutchai and Steen (1971); Lomholt (1976) and Tullett and Board (1976).

Although we present evidence that changes in the limiting membrane cause a decrease in oxygen resistance, previous workers (Kutchai and Steen, 1971; Lomholt, 1978; Tullett and Board, 1976; Kayar et al. 1981) have centred their discussions on the contribution of a water film to the membranes' resistance to oxygen flux. Thus Kayar et al. (1981) calculated that this film is 63 µm thick in eggs incubated for less than 4 days but only 0.6 µm in those incubated for 12-17 days and considered it plausible that this thinning occurred in the inner shell membrane alone. Not only are the discussions of a water film based on inference, they fail to take into account the microstructure of the shell membranes and the possible physico chemical contribution of the mantles on the individual membranes. As is evident in Fig. 3a, there is a progressive diminution in the inter-fibre distances and fibre diameters from the outside to the inside of the inner shell membrane. It would seem reasonable to assume from the studies of the behaviour of water in soils (Hillel, 1980) that if there was a progressive thinning of a water film in the membrane then the resistance to evaporative loss would increase, providing the mantles did not in some way modify the physical properties of the water connecting them. There was no evidence of such an increase in Fig. 2.

Moreover if drying alone was responsible for the decrease in oxygen resistance noted in this study and those quoted above, why did it not occur in infertile eggs incubated alongside fertile ones (Fig. 1)?

The conclusion of Kayar et al. (1981) that the inner shell membrane accounts for 88% of the initial resistance of the egg's integument to oxygen diffusion would appear to be in need of amendment in the light of the results discussed above. In practice their estimate that it contributes only 12% of the total resistance in eggs incubated for more than 4 days is probably correct, the 66% of the resistance noted in freshly laid fertile eggs and transiently in those incubated for 4 days or less being due to the limiting membrane. In summary, therefore, the impediments to oxygen diffusion across the cuticle, porous calcitic eggshell and the underlying membranes can be considered as resistances in series (Fig. 6). Of the 6 potential resistances shown in this diagram, that due to the limiting membrane (No. 6) appears to be temporal only in incubating eggs.

As the evidence presented in this paper has identified the limiting membrane as an important resistance to oxygen diffusion, it is pertinent to consider mechanisms that diminish its resistance through propagating crack formation. In planning the present studies, attention was directed at the possibility of time-induced changes in the thickness of the mantles of the fibres, it being assumed that a reduction in mantle thickness would enlarge the spaces between fibres. It is known, moreover, that the space filled by the glycoprotein cortex of the bacterial endospore (Gould and Pring, 1975) is determined by the nature of the cations involved in cross-linking. Thus the cortex occurs in the expanded form when K^+ and Na^+ are present but in the contracted form with Ca^{2+}. Although we demonstrated a diminution in the Na^+ and K^+ content (Fig. 5) of the shell membranes of incubated fertile but not

infertile eggs, there was no corresponding change in the dimensions of the mantles. Moreover we are of the opinion that changes in the cation content of the mantles resulted from those in the concentration of these cations in underlying albumen. According to Tullett and Board (1976) the latter changes are associated with the transfer of water from the albumen during the formation of the sub-embryonic fluid and we speculate that cation loss from the shell membranes reflects transfer caused by a diffusion gradient being established between the shell membranes and the albumen. As there was no evidence of change in the size of the fibre mantles with incubation, the cracks in the limiting membrane cannot be attributed to mechanical stresses caused by changes in the support provided by the inner shell membrane.

We speculate that the cracks occurring in the limiting membrane are caused by mechanical stress due to egg turning. Future studies are needed to discriminate between stress due to the membrane being rubbed with albumen thickened as a consequence of water loss to the sub-embryonic fluid or the developing chorioallantois.

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REFERENCES

ANON. (1973). The analysis of agricultural materials. A.D.A.S. Technical Bulletin No.27. H.M.S.O., London.

Baker, J.R. and D.A. Balch (1962). A study of the organic material of hen's egg shell. Biochemical Journal 82 : 352-361

Bellairs, R. and A. Boyde (1969). Scanning electron microscopy of the shell membranes of the hens' egg. Z. Zellforsch. Mikrosk. Anat. 96 : 237-249.

Board, R.G. and G. Love (1980). Magnesium distribution in avian egg-shells. Comparative Biochemistry Physiology 66A : 667-672.

Bond, G.M. (1980). Avian eggshells and the hatching process. Ph.D. Thesis, University of Bath.

Cooke, A.S. and D.A. Balch (1970). Studies of membrane, mammillary cores and cuticle of the hen egg shell. British Poultry Science 11 : 345-352.

Crombie, G., R. Snider, B. Faris and C. Franzblau (1981). Lysine-derived cross-links in the egg shell membrane. Biochimica et Biophysica Acta 640 : 365-367.

Froix, M.F., J. Pochan, A. Goedde and D.L. Hinman (1977). A dynamic characterization via relaxation techniques of the shell membranes of the domesticated fowl (Gallus/gallus (L.)). Polymer 18 : 1213-1218.

Gould, G.W. and G.J. Pring (1975). Heat resistance of bacterial endospores and concept of an expanded osmoregulatory cortex. Nature (London). 258 : 402-405.

Harris, E.D. J.E. Blount and R.M. Leach, Jr. (1980). Localization of lysyl oxidase in hen oviduct : Implications in eggshell membrane formation and composition. Science 208 : 55-56.

Hillel, D. (1980). Fundamentals of soil physics. Academic Press, London.

Kayar, S.R., G.K. Snyder, G.F. Birchard and C.P. Black (1981). Oxygen permeability of the shell and membranes of chicken eggs during development. Respiration Physiology 46 : 209-221.

Kutchai, H. and J.B. Steen (1971). Permeability of the shell and shell membranes of hens' egg during development. Respiration Physiology 11 : 265-278.

Leach, R.M. and R.B. Rucker (1978). Studies on the major protein component of egg shell membranes. Poultry Science 67 : 1151.

Leach, R.M., R.B. Rucker and G.P. Van Dyke (1981). Egg shell membrane protein: A non-elastin desmosine/isodesmosine - containing protein. Archives Biochemistry Biophysics 207 : 353-359.

Leeson, T.S. and C.R. Leeson (1963). The chorio-allantois of the chick. Light and electron microscopic observations at various times of incubation. Journal of Anatomy (London) 97(4) : 585-595.

Lomholt, J.P. (1976). The development of the oxygen permeability of the avian egg shell and its membranes during incubation. Journal of Experimental Zoology 198 : 177-184.

Skalinsky, E.I. and V.F. Kondalenko (1963). Electron microscopic studies of the chick chorio-allantois during embryogenesis. Acta Morphologica 12 : 247-259.

Starcher, B.C. and G.S. King (1980). The presence of desmosine and isodesmosine in eggshell membrane protein. In International Review of Connective Tissue Research (eds. D.A. Hall and D.S. Jackson), Vol.8, pp.53-55. Academic Press, New York and London.

Temple, C.F. and J. Metcalfe (1970). The effects of increased incubator oxygen tension on capillary development in the chick chorioallantois. Respiration Physiology 9 : 216-233.

Tullett, S.G. and R.G. Board (1976). Oxygen flux across the integument of the avian egg during incubation. British Poultry Science 17 : 441-450.

Wangensteen, O.D., D. Wilson and H. Rahn (1970/71). Diffusion of gases across the shell of the hen's egg. Respiration Physiology 11 : 16-30.

Wedral, E.M., D.V. Vadehra and R.C. Baker (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of Gallus gallus. Comparative Biochemistry Physiology 47B : 631-640.

Fig. 1. The rate of oxygen transfer across the shell and shell membranes of the infertile (closed circles and solid line) and fertile (open circles and broken line) eggs of the domestic hen. Each symbol is the result obtained with one egg incubated (37.5°C) with turning for the period shown.

Fig. 2. The weight loss from twelve fertile eggs of the domestic hen during incubation (37.5°C) with turning for 14 days. Open circles, means; bars, range.

Fig. 3. Electron microscope examination of the shell membranes of the eggs of domestic hens. (a) Scanning electron micrograph of a radial section of shell membranes prepared by freeze fracture and freeze drying; O, outer shell membrane (thick fibres), I, inner shell membrane (thin fibres), and LM, limiting membrane (electron dense material) bar marker, 10 μm. (b) Transmission electron micrograph of a thin radial section (stained with ruthenium red) of the fibres in the shell membrane; c, cortex; m, mantle and bar marker, 1.0 μm.

Fig. 4. Scanning electron micrograph of the limiting membrane of the eggs of domestic hens incubated (37.5°C) with turning. (a) The electron dense limiting membrane in infertile and fertile eggs incubated for up to 4 days appeared to be intact. (b) and (c) Cracks and, subsequently, large gaps appeared in the limiting membrane (M), exposing the underlying outer membrane (F) of fertile hen's eggs in which there was a marked change (Fig.1) of the K_{O_2} of the shell and shell membranes. Bar marker, 10 μm.

Fig. 5. The concentration of calcium, magnesium, potassium and sodium in the shell membranes of infertile (open squares) and fertile (open circles) eggs of the domestic hen incubated (37.5°C) with turning for the times indicated. Each symbol represents the average result of 4 replicates obtained from a total of 24 eggs.

Fig. 6. The diffusion pathway through the shell and shell membranes of the egg of the domestic hen viewed as resistances in series: 1, boundary layer; 2, cuticle; 3, pore canal; 4, outer shell membrane; 5, inner shell membrane, and 6, limiting membrane.

Figure 1

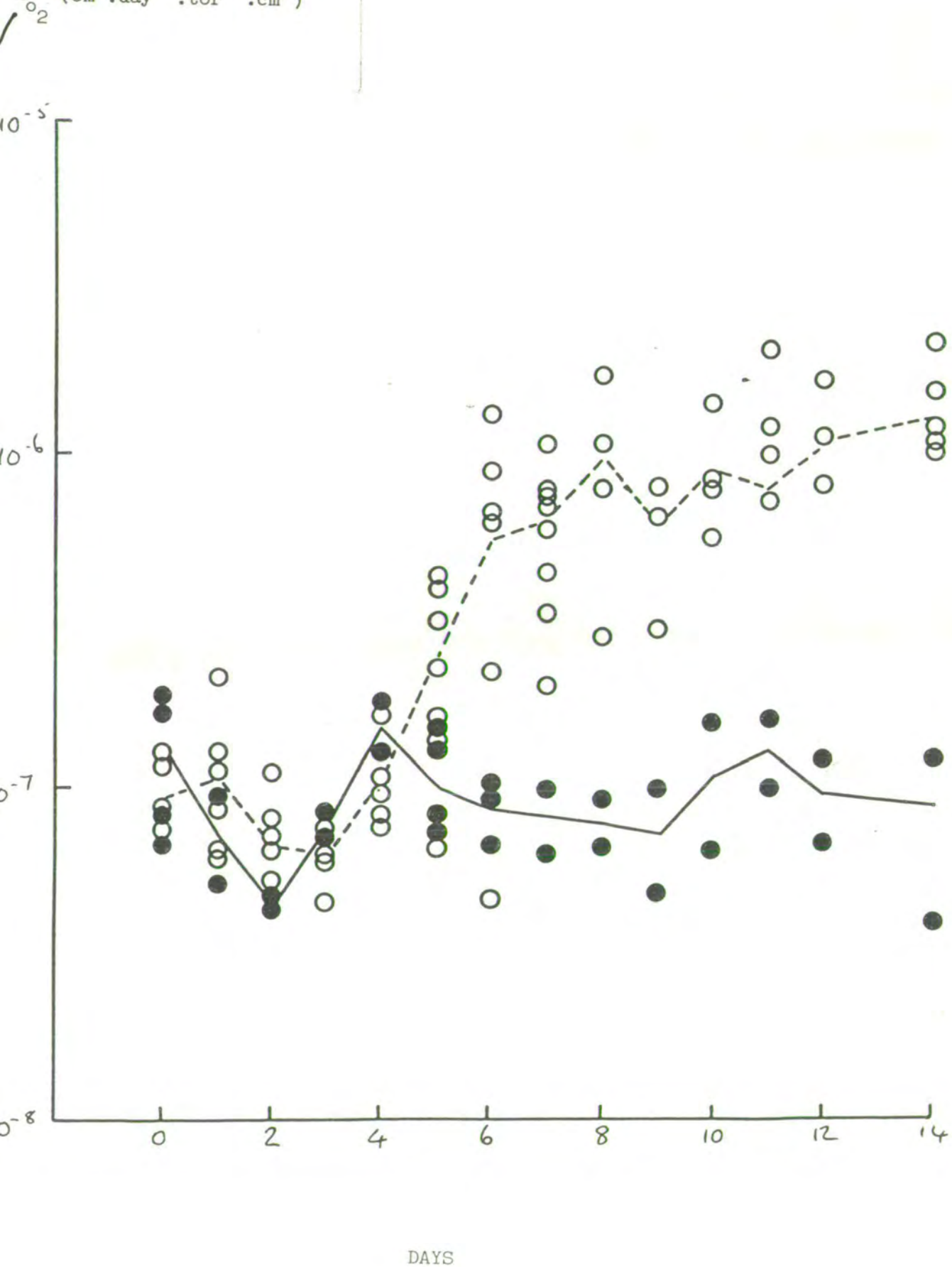
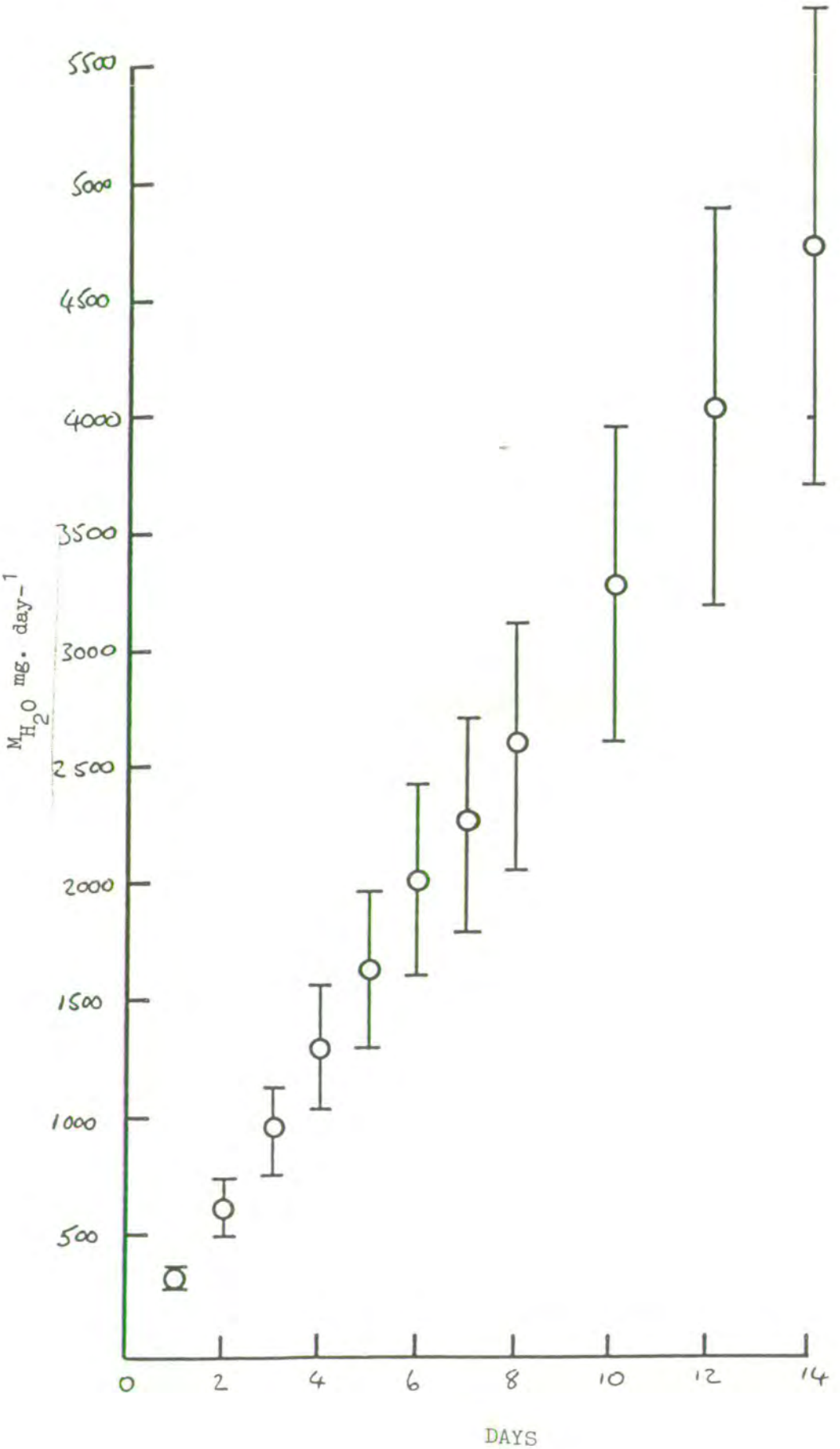


Figure 2


5500
5000
4500
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3000
2500
2000
1500
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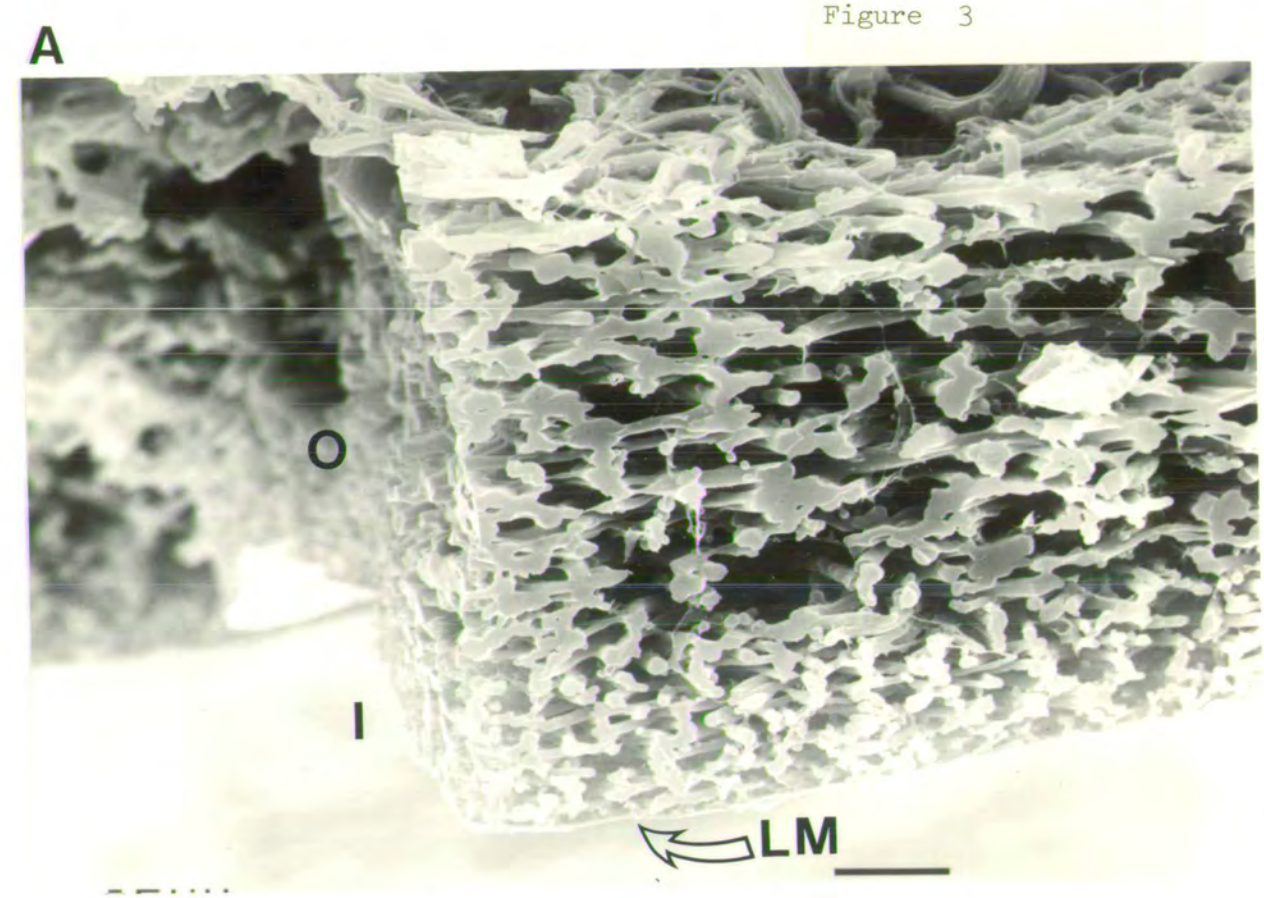
500
M_{H_2O} mg. day^{-1}
0
2
4
6
8
10
12
14
DAYS

Figure 3



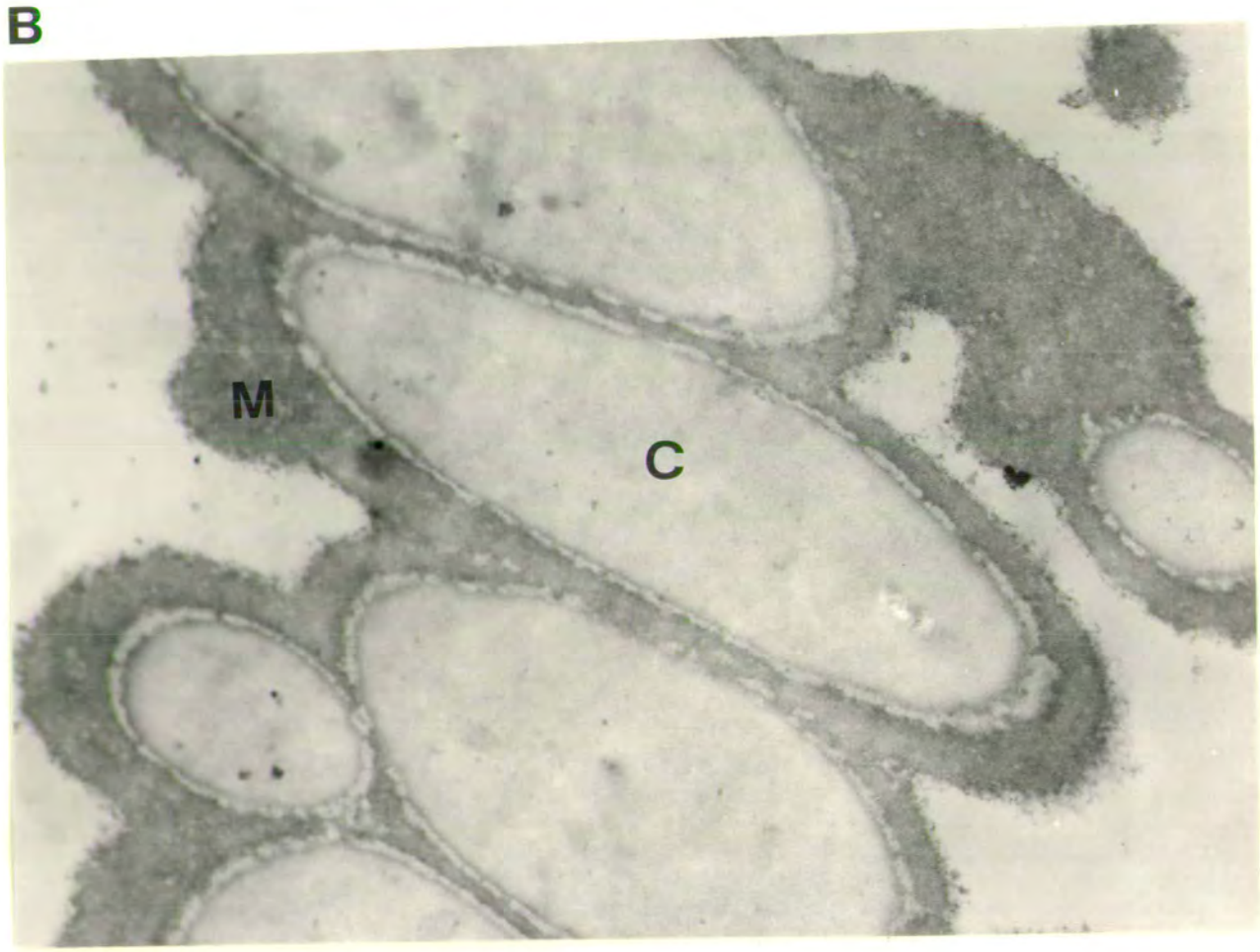
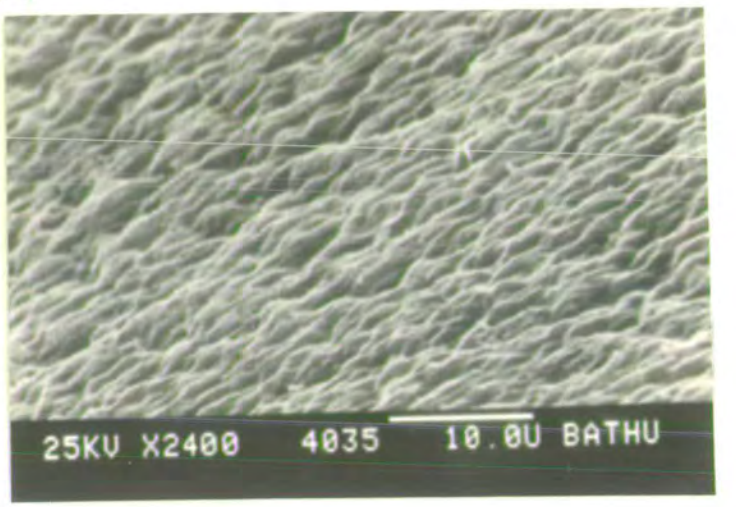


Figure 4

A



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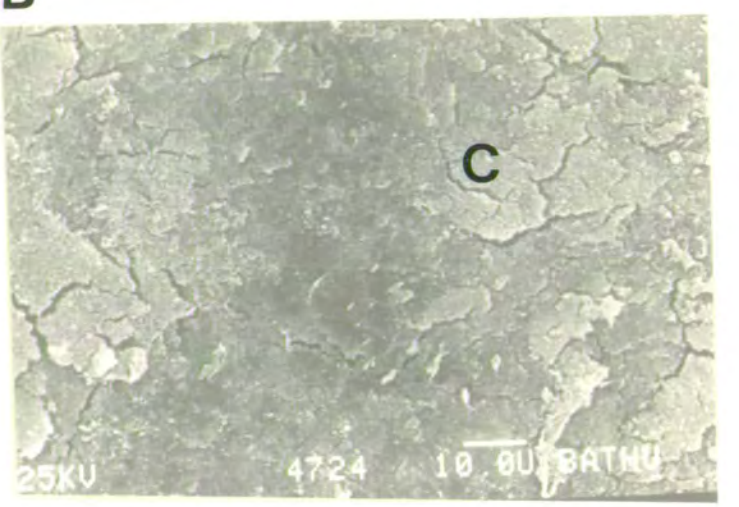


C

Figure 4

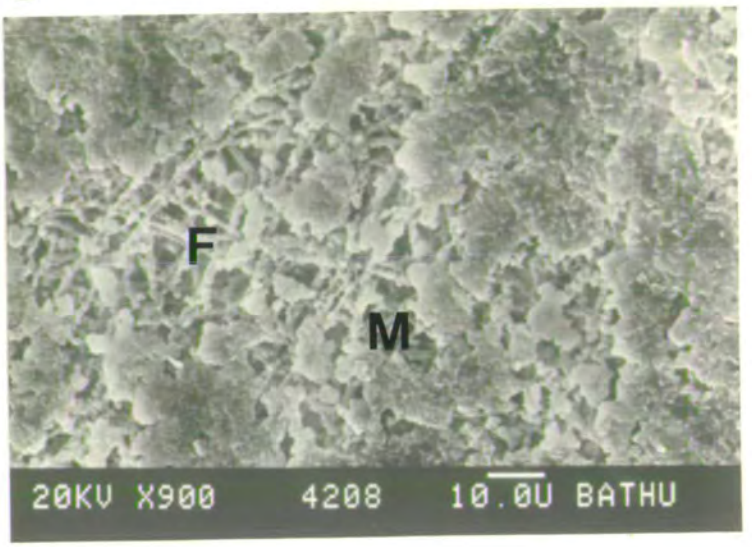


Figure 5

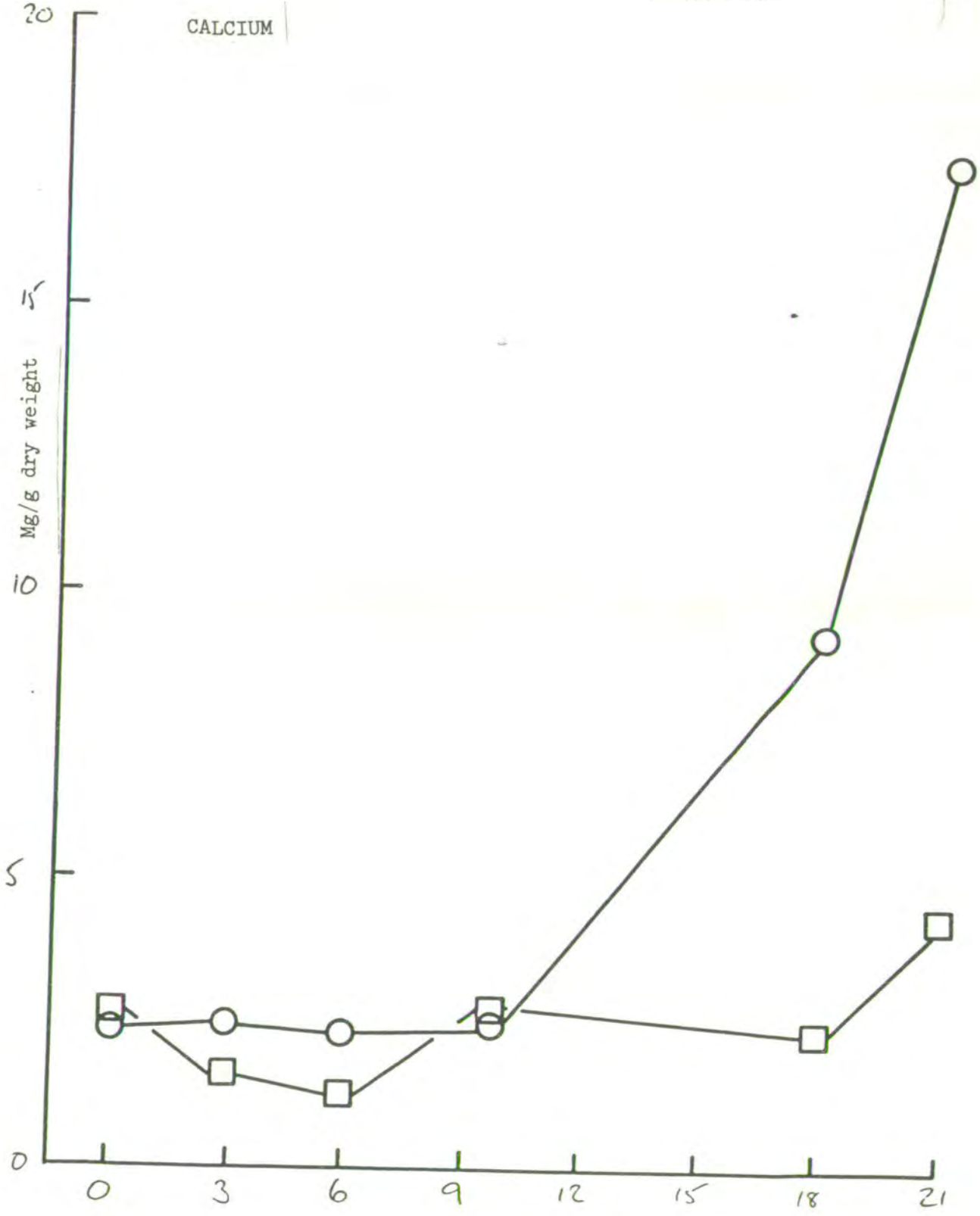
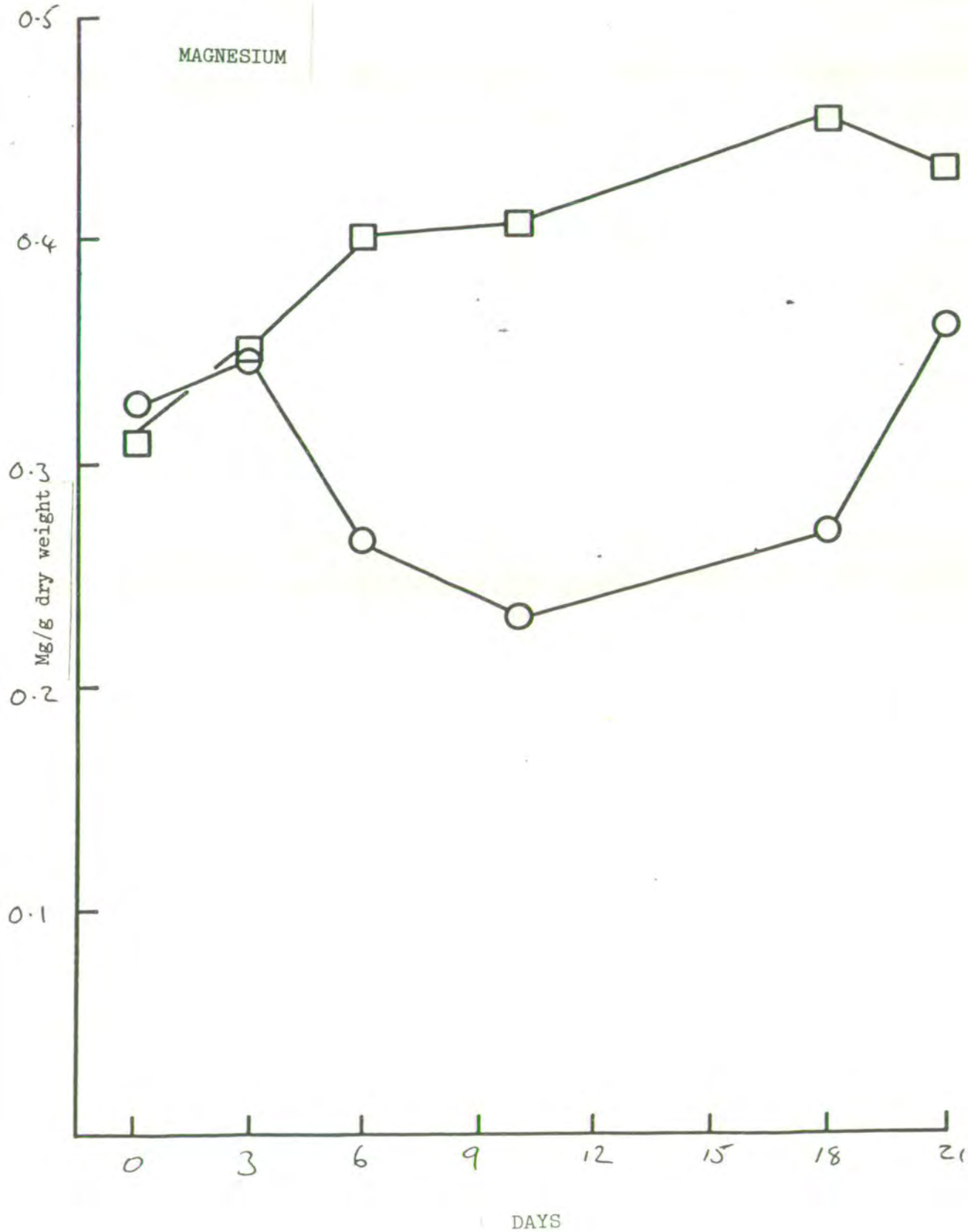


Figure 5



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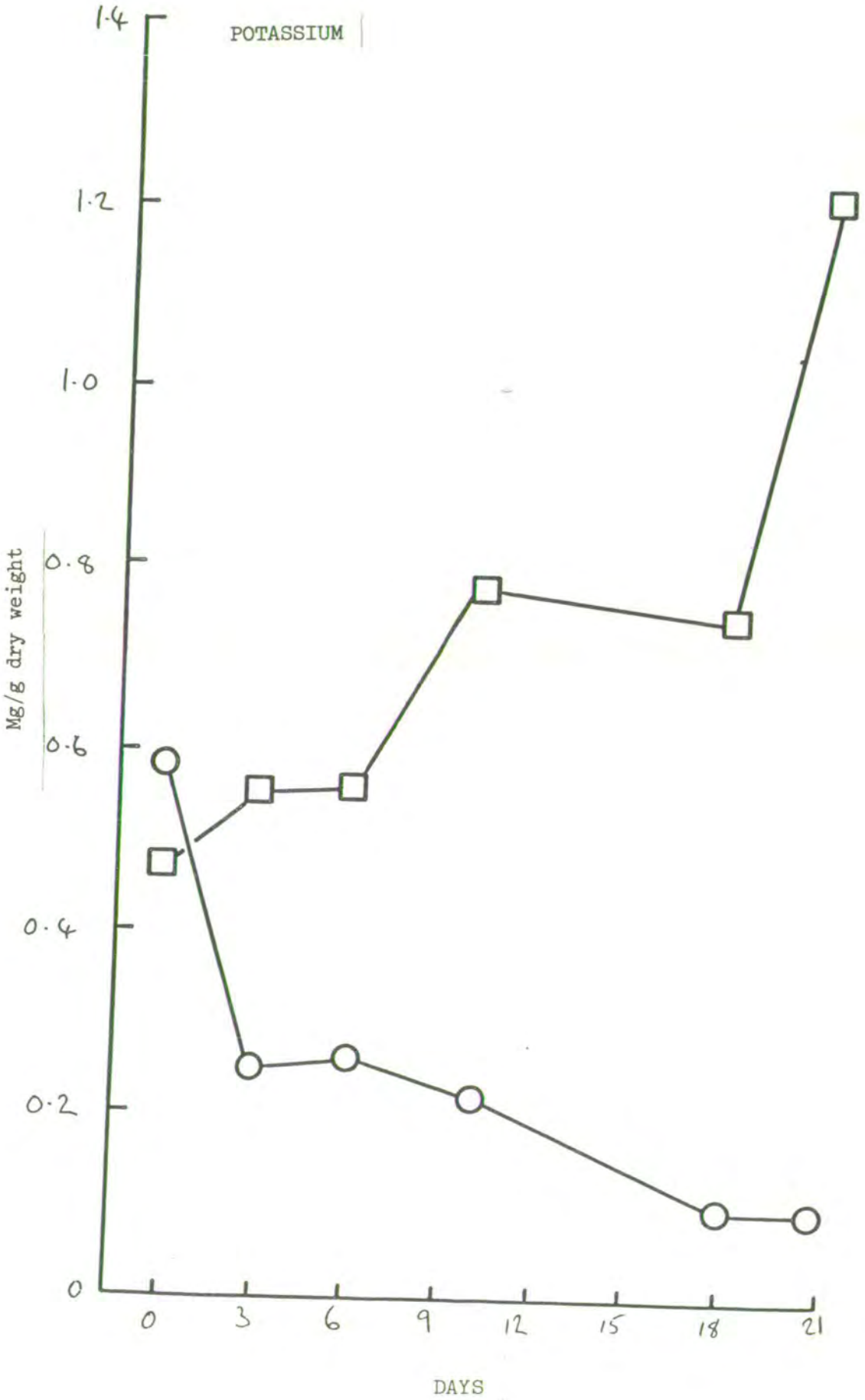

POTASSIUM
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Mg/g dry weight
0
3
6
9
12
15
18
21
DAYS

Figure 5

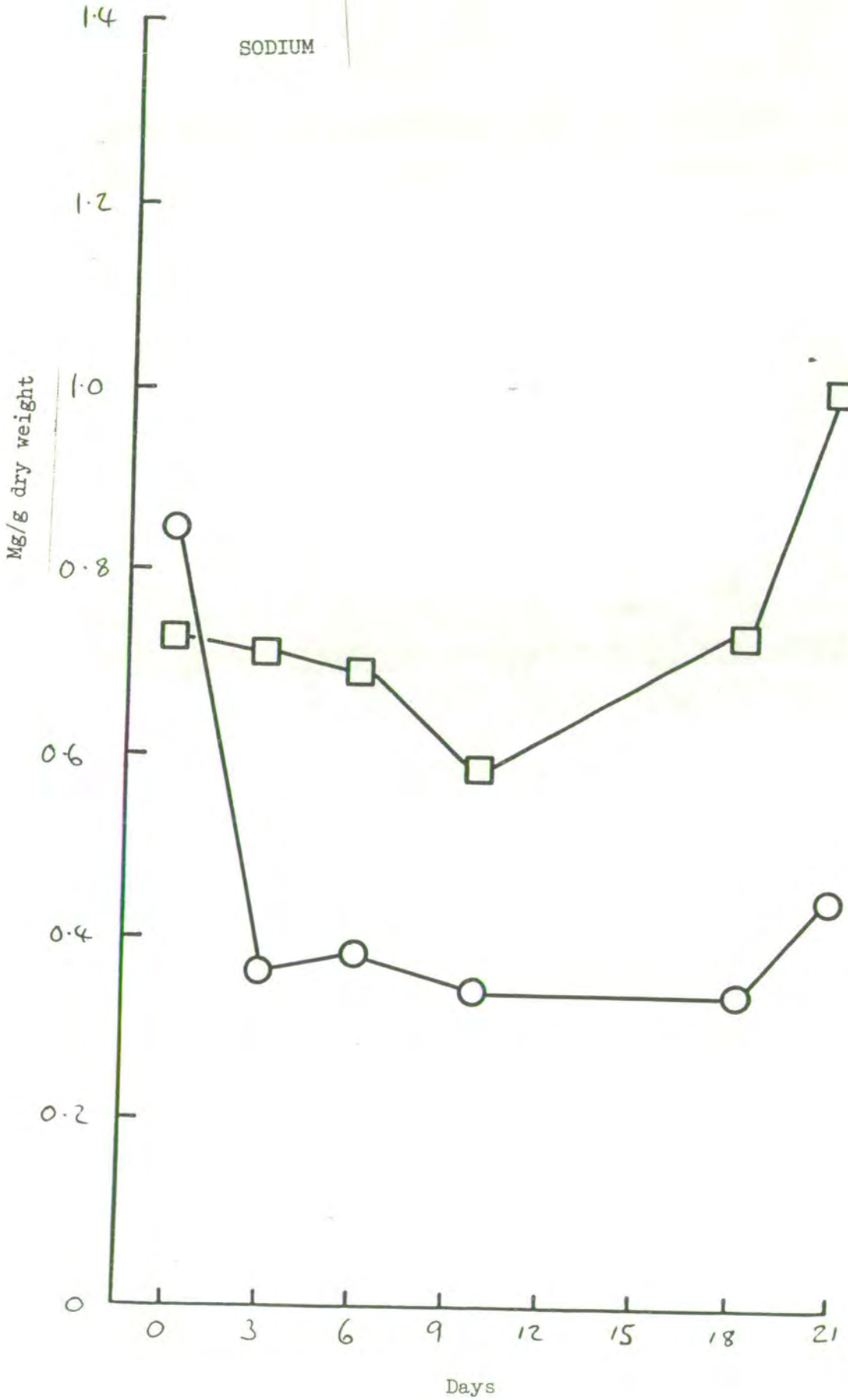
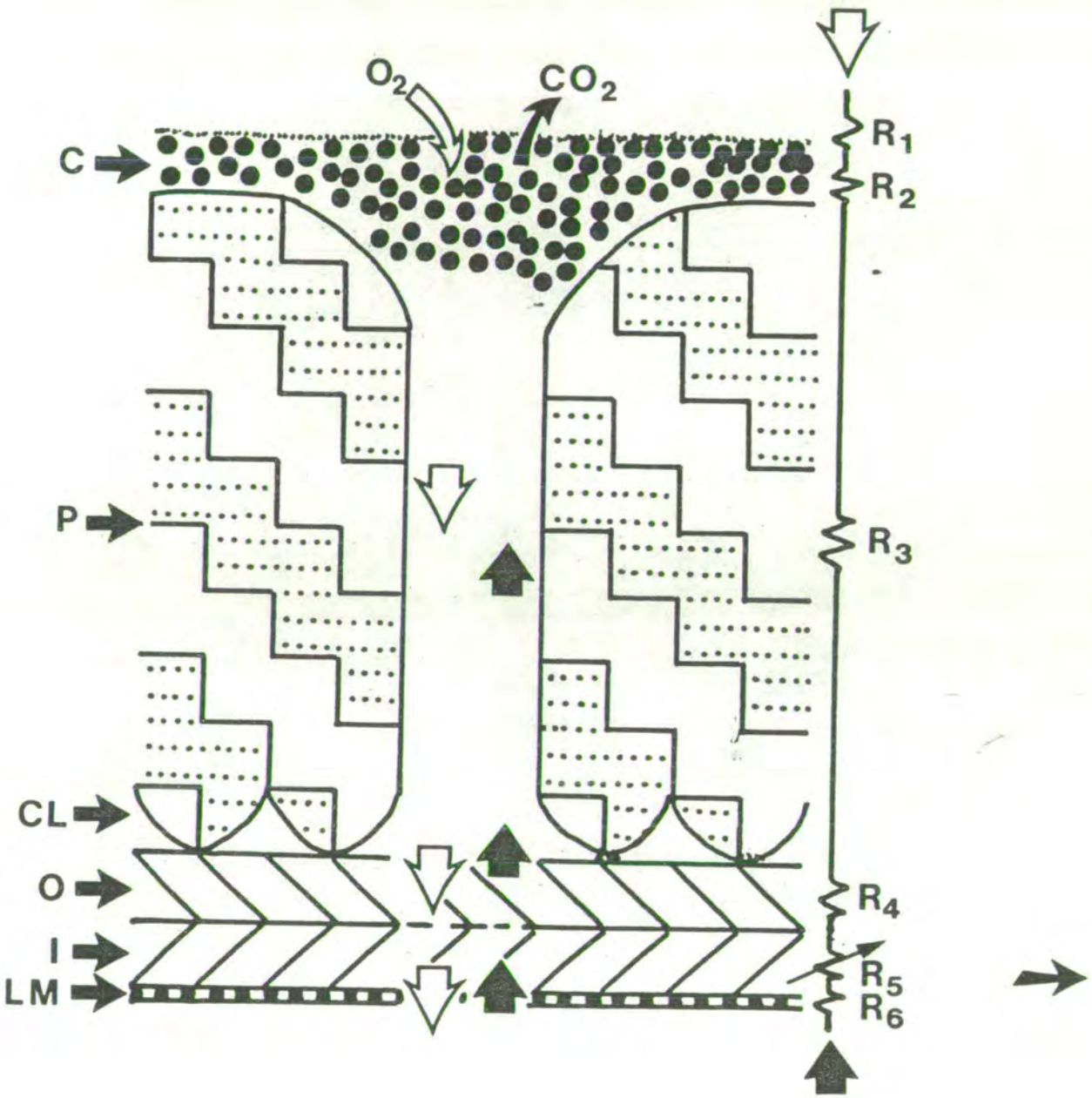


Figure 6



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MICROSTRUCTURE, WATER RESISTANCE AND WATER REPELLENCY OF THE PIGEON EGG SHELL

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SYNOPSIS

The shell of the pigeon egg has no cuticle. It is water repellent but the pores are flooded easily with water when a force is applied. It is proposed that in considerations of the interaction of water and the shells of eggs, the terms water repellency and water resistance should be used in preference to the imprecise term, water-proofing.

INTRODUCTION

It was noted by Board and Halls (1973*a*, *b*) that the cuticle impedes liquid and particle penetration of the egg shell of the domestic hen (*Gallus domesticus*), mallard (*Anas platyrhynchos* L.) and guinea fowl (*Numidia meleagris*). When discussing the biological implications of their observations, these authors surmised that, in addition to contributing to the antimicrobial defence, the cuticle contributed to the water-proofing of the shell. Thus the cuticle helped to prevent asphyxiation of the embryo which would ensue if the shell became waterlogged as a result of the flooding of the pores and the spaces between the cones and shell membranes. It has been noted subsequently that the shell of pigeon eggs does not have a cuticle. Studies of these shells have shown that the term, water-proofing, is too imprecise and that the terms, water repellent and water resistant, should be used when describing the interaction of water and the avian egg shell. The evidence in support of this proposal is the subject of this communication.

MATERIALS AND METHODS

The eggs

The pigeon eggs were obtained from: (*a*) a small flock of fan-tails which roosted in a hut, (*b*) a diverse collection of fan-tails, racing pigeons and their progeny which roosted in the rafters of a house in which laying hens were kept in batteries, and (*c*) a flock of racing pigeons. Breeding success was high amongst the latter two sources.

Scanning electron microscopy

A scanning electron microscope (SEM; Stereoscan S4, Cambridge Instrument Co. Ltd) was used to study the microstructure of the shell. Pieces of shell were cemented on to aluminium stubs with colloidal silver and coated (under vacuum) with gold : palladium.

RESULTS

Microstructure

The surface of pigeon egg shell was not stained by Edicol pea green (Edicol Supra Pea Green H; ICI, Hexagon House, Blakeley, Manchester), a dye that was used by Board and Halls (1973*a*, *b*) to demonstrate cuticle on the egg shells of domestic hens, quail, mallard and guinea fowl. No fine structure could be seen when the surface of pigeon eggs was examined microscopically with reflected light. With transmitted light, the pores appeared as areas of brilliance against a dull grey background. In thin, radial sections of decalcified shell (calcium removed with EDTA containing formalin) which were stained by Masson's method, there was no discrete, heavily stained material on the outer surface of the shell of pigeon eggs. With the egg shells of domestic hen, cuticle was easily demonstrated by this method. Moreover, no covering material was seen on the outer surface of the shells of pigeon eggs which had been embedded in plastic and ground to a thinness which allowed radial sections to be studied with transmitted light.

When pigeon eggs were submerged in water under vacuum, large gas bubbles were immediately released from all parts of the shell. This situation is in contrast to that of hens' eggs where a vacuum causes a few pores to discharge gas initially and, subsequently, for many of the pores to produce a bubble which gives a frothy appearance to the shell (Board and Halls, 1973*a*). Examination of pigeon egg shell with the SEM showed that the outer orifice of the pores was open (Plate-Fig. 1). Some of the pore canals contained debris (Plate-Fig. 2) but this did not appear to be arranged in a manner that would provide an effective plug. The wall of the pore canal may be covered with a film, a possibility which is borne out by comparison of the fine structures in Plate-Fig. 2 with that of a piece of shell (Plate-Fig. 3) which had been scoured for 5 min in boiling NaOH (5% w/v). The pore canal in the latter is seen to contain a large number of pits. The surface of the shell (Plate-Fig. 4) contained pits, and these were seen in the face of the shell exposed by a radial fracture (Plate-Fig. 5) and in the cones (Plate-Fig. 6) on the inner surface of the shell.

Water repellency

To the naked eye, the clean surface of the pigeon egg shell has the appearance of porcelain. When a drop of water was placed carefully on to the shell, it remained as a drop with an angle of *ca.* 60° between it and the surface of the shell. Tapping the egg caused violent movement of the liquid within the drop but a sharp jolt was required to overcome the tension at the interface of the shell and the periphery of the drop. Water was shed quickly when drops were allowed to fall from an inch or so onto a piece of inclined shell. On stopping the flow of drops, there remained on the shell a film of moisture which dried rapidly.

Water resistance

When pigeon eggs were sprayed with a 1% w/v) aqueous solution of Edicol pea green from a watering can, the rose of which was held about 2 m above the eggs, the pores were flooded in those parts of the shell upon which the drops fell. The open, cone-shaped orifice of many pores (Plate-Fig. 2) is obviously not adapted

to dissipate the energy in falling drops of water. Such a spray did not cause flooding of the pores in the shell of the hen's egg unless the cuticle had been removed with a neutralised (pH 7·5) solution of ethylenediaminetetracetic acid (5% w/v). Even with such cuticle-less shells, only a few pores were flooded. The violent pouring of an aqueous suspension of an encapsulated pigment (Terasil Brilliant Blue 2G liquid, Ciba-Geigy) from a beaker on to pigeon eggs caused the pores to be breached by the particles which were about 1-5 μm diameter.

TABLE 1

Water uptake by pigeon eggs

Treatment	Egg no.	Weight change (mg/egg)	Treatment	Egg no.	Weight change (mg/egg)
Eggs at room temperature were immersed in iced water for 15 min.	1	+148·2	Eggs allowed to return to room temperature were immersed in water at room temperature for 15 min.	1	+77·9
	2	+179·3		2	+70·6
	3	+21·7		3	+66·8
	4	+134·5		4	+54·5
	5	+123·3		5	+40·6
	6	+93·8		6	+89·7
	7	+139·7		7	+52·0
	8	+169·3		8	+19·1
	9	+183·8		9	+74·9
	10	+151·5		10	+69·6
	11	+129·0		11	+104·3
	12	+121·1		12	+56·2
	13	+140·8		13	+54·9
	14	+168·6		14	+141·1
Eggs at room temperature were immersed in water at room temperature for 15 min.	15	+1·1	Eggs at room temperature were immersed in iced water for 15 min.	15	+188·7
	16	−1·5		16	+211·4
	17	−2·5		17	+180·9
	18	+1·0		18	+207·9
	19	−0·5		19	+491·4
	20	+3·7		20	+141·5
	21	−0·6		21	+184·8
	22	+0·3		22	+173·0
	23	+13·2		23	+160·2
	25	+3·5		25	+88·6
	26	−0·1		26	+157·4
	27	+16·8		27	+206·2
	28	+2·1		28	+177·3

Additional evidence that the pores offer negligible resistance to water under force came from studies in which warm (room temperature) pigeon eggs were immersed in iced water (Table 1). The contraction of the eggs caused the pores to be flooded, and water uptake was appreciable (*ca.* 90-180 mg/egg). There was only a slight increase in weight of some of the eggs immersed in water at room temperature. The data shown in Table 1 also indicate that the negligible water resistance of the pores can be negated permanently. Thus when eggs which had been immersed in chilled water were placed subsequently in water at room temperature, they absorbed more water than those which had been exposed initially to water at room temperature.

Imposing momentarily a positive pressure (0·07 kg/cm^2) to the head space of a closed container in which eggs were immersed in a solution of Edicol pea green resulted in the eggs absorbing water (Table 2). Likewise, the abrupt breaking of a vacuum in a sealed container holding pigeon eggs in water caused the eggs (Table 2)

to increase in weight. Because of the difficulties encountered in getting large numbers of pigeon eggs, it was not possible to ascertain the minimum pressure required to overcome the water resistance of the pores.

TABLE 2

Water uptake by pigeon eggs

Treatment	Egg no.	Weight change (mg/egg)
Eggs immersed in water for 10 min, a vacuum drawn, 2 min, and eggs soaked for further 3 min.	1	+502·5
	2	+280·8
	3	+455·8
Eggs immersed in water for 5 min, a vacuum applied for 2 min, and eggs soaked for a further 8 min.	4	+704·2
	5	+277·6
	6	+279·4
Eggs immersed in water for 15 min.	7	+34·3
	8	+31·0
	9	+56·9
	10	+33·5
Positive pressure applied to eggs for 1 min, eggs allowed to soak for 15 min.	11	+408·0
	12	+246·2
	13	+123·5
	14	+300·8
	15	+130·2
	16	+171·8
Eggs soaked for 15 min in water at room temperature.	17	+5·9
	18	−9·8
	19	+5·0
	20	+0·8
	21	−0·6
	22	−1·2

DISCUSSION

Although this study was concerned with pigeon eggs only, the results merit consideration in the broader context of the interaction of water and the shell of the avian egg. In another discussion (Board and Halls, 1973*a*), it was claimed that the cuticle on the shell of the hen's egg was associated with water-proofing. It would now seem that two separate but, possibly, complementary properties are involved in the prevention of water-logging of the shell, namely water repellency, probably a function of the microstructure and chemical composition of the exposed shell surface, and water resistance, a function of the gross structure of the shell – the capping and or plugging of pores by cuticle. From a biological viewpoint, the need for and contribution made by these properties may well be determined by the nesting site of birds. Thus the eggs of ground-nesting species (the domestic hen, quail, pheasant, coot, moorhen, mallard, domestic duck, red legged partridge and guinea fowl) examined by the author have cuticle and a marked water resistance. Some, such as the eggs of mallards, have well-developed properties of water repellency also. Tyler (1969) has also noted that a well-developed cuticle is present on

the shells of the eggs of many species of ground-nesting birds. It is tempting to speculate that the cuticle is a feature of eggs which are exposed to rain; it provides a resistance which is not overcome by the energy contained in drops of falling water. Water repellency, on the other hand, provides a means whereby the pores are protected from flooding merely by contact with a wet surface.

The water repellency of the egg shell may well be accentuated during brooding by preening oils being smeared over its surface. Thus we have noted that the water repellency of pheasant eggs taken from a brooding bantam is more pronounced than of those examined immediately following oviposition.

The present study provided information which is of interest in the context of egg washing machines. From a theoretical viewpoint, successful cleansing of dirty shells could be expected to result from a negation of the water repellency of the shell, a process that would be assisted by the use of a surface active agent such as a detergent. There is an obvious requirement to prevent the water resistance of the shell being overcome. Following the observations (Haines and Moran, 1941) that contraction of warm eggs in cold water causes the translocation of water and micro-organisms across the shell, emphasis has been given to maintaining the temperature of the egg below that of the washing and rinsing water (Brant *et al.*, 1966; Büchli, 1967). As far as can be ascertained, little attention has been given to the pressure of the water used, particularly for rinsing. Studies of the microstructure of naturally cuticle-less eggs of the domestic hen (Board, 1974) have shown that the pores are naked. From the observations of Board and Halls (1973*a*), it can be presumed that such eggs have limited resistance to water and that they could be infected if an egg washing machine caused force to be applied to a wet shell.

ACKNOWLEDGEMENTS

We wish to thank Professor G. V. T. Matthews, Mr P. Hunt and Mr and Mrs Hoddinott for gifts of pigeon eggs; Dr V. D. Scott and Mr H. R. Perrott for the use of the SEM, and Mr D. Bennett for photographic assistance.

REFERENCES

BOARD, R. G. (1974). The microstructure of the cuticle-less shells of the eggs of the domestic hen. *Br. Poult. Sci.*, (in press).

BOARD, R. G. AND HALLS, N. A. (1973*a*). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poult. Sci.*, **14**: 69-97.

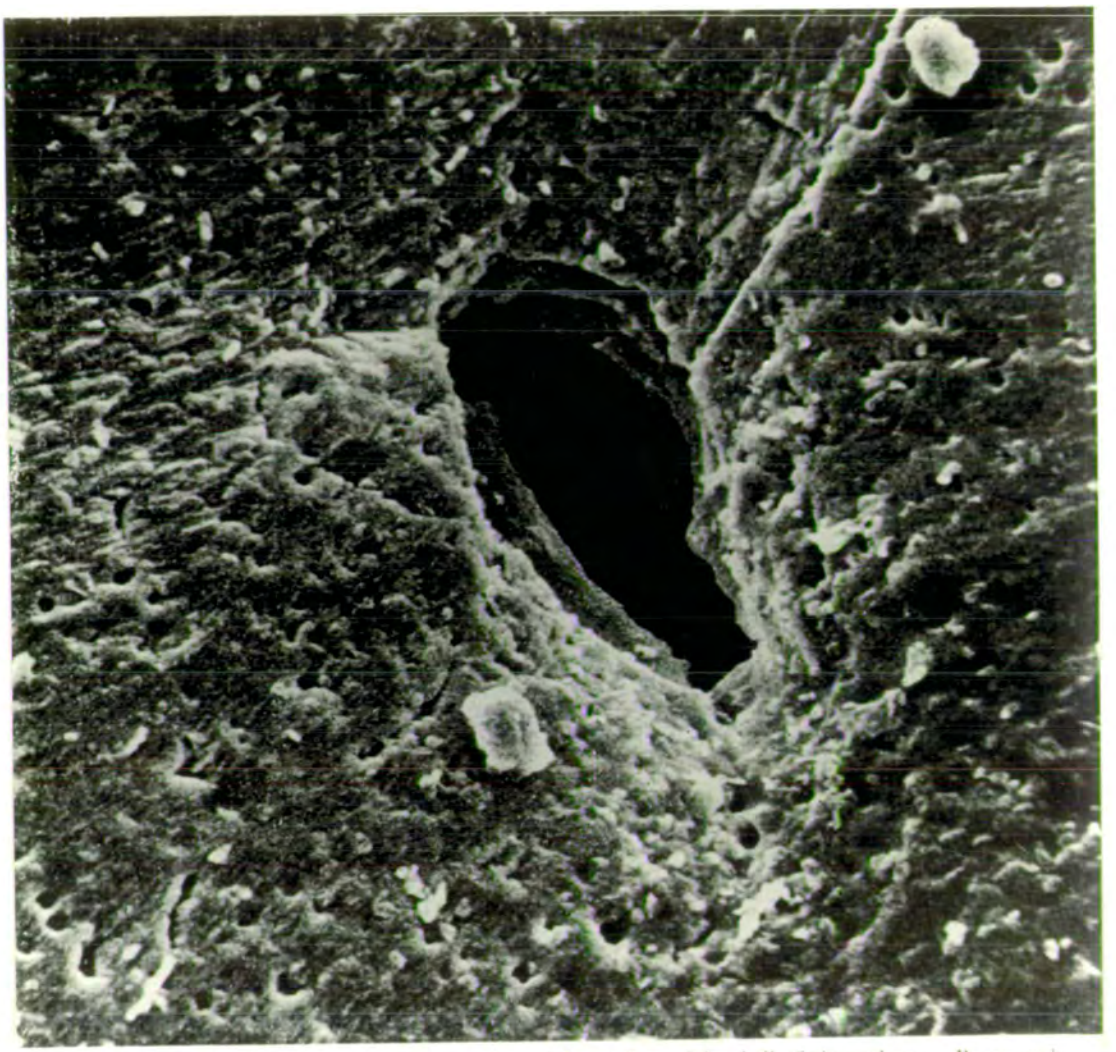
BOARD, R. G. AND HALLS, N. A. (1973*b*). Water uptake by eggs of mallard and guinea fowl. *Br. Poult. Sci.*, **14**: 311-314.

BRANT, A. W., STARR, P. B. AND HAMANN, J. A. (1966). *Poultry Tribune*, 65.

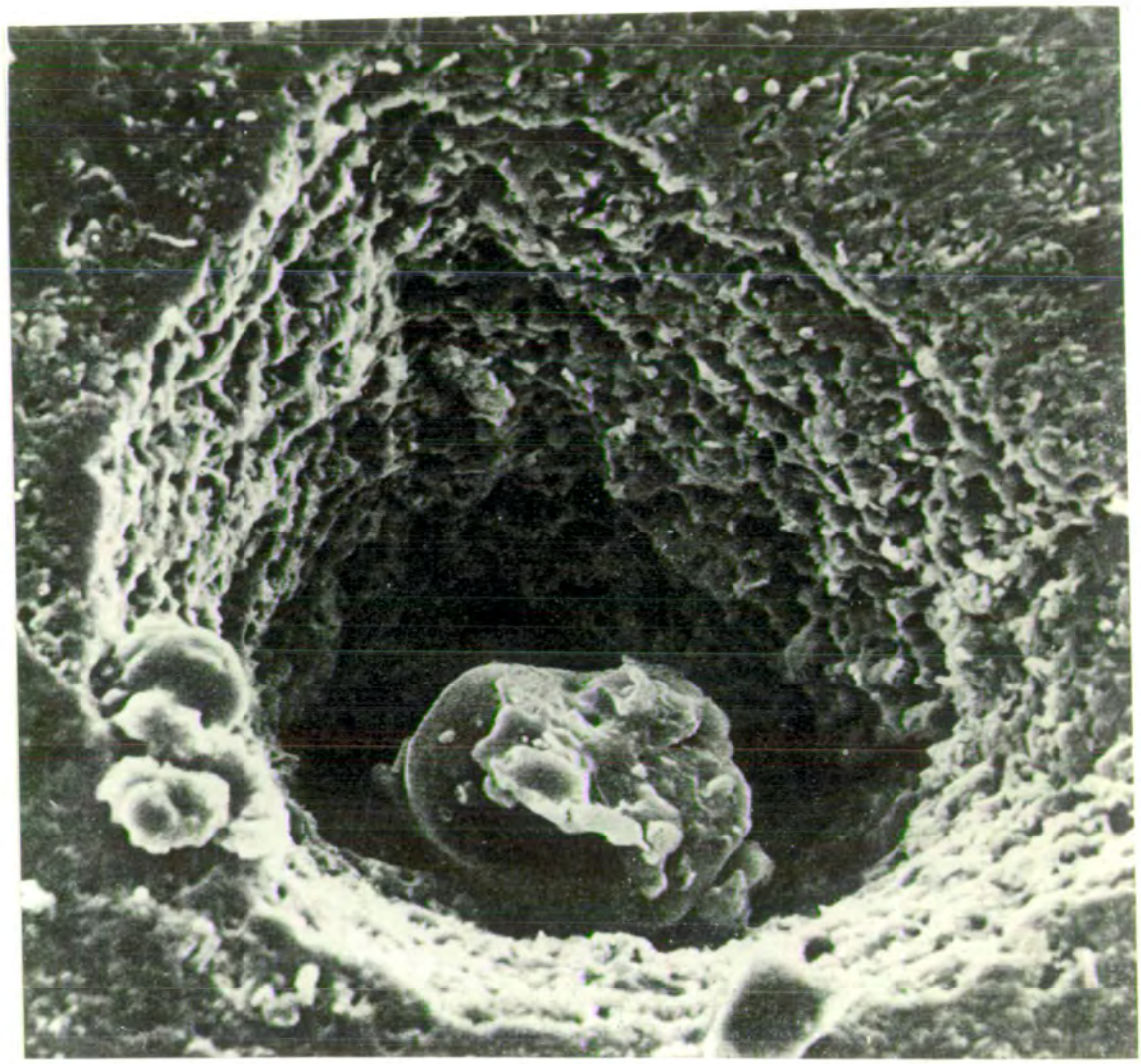
BÜCHLI, K. (1967). De invloed van het wassen op de microbiologische houdbarer—heid van het ei. *Instituut voor der Pluimveeteelt "Het Spelderholt"—Beekbergen Mededeling No.* 145.

HAINES, R. B. AND MORAN, T. (1941). Porosity of, and bacterial invasion through the shell of the hen's egg. *J. Hyg., Camb.*, **40**: 453-461.

TYLER, C. (1969). Avian eggs: their structure and characteristics. *Int. Rev. gen. exp. Zool.*, **4**: 82-127.



PLATE—FIG. 1.—Scanning electron microscope view of the surface of the shell of pigeons' eggs. Beam angle, 12·5°; KV, 10.



PLATE—FIG. 2.—Pores in the shell of pigeons' eggs as seen with the Scanning Electron Microscope. Beam angle, 45 ; KV, 10.

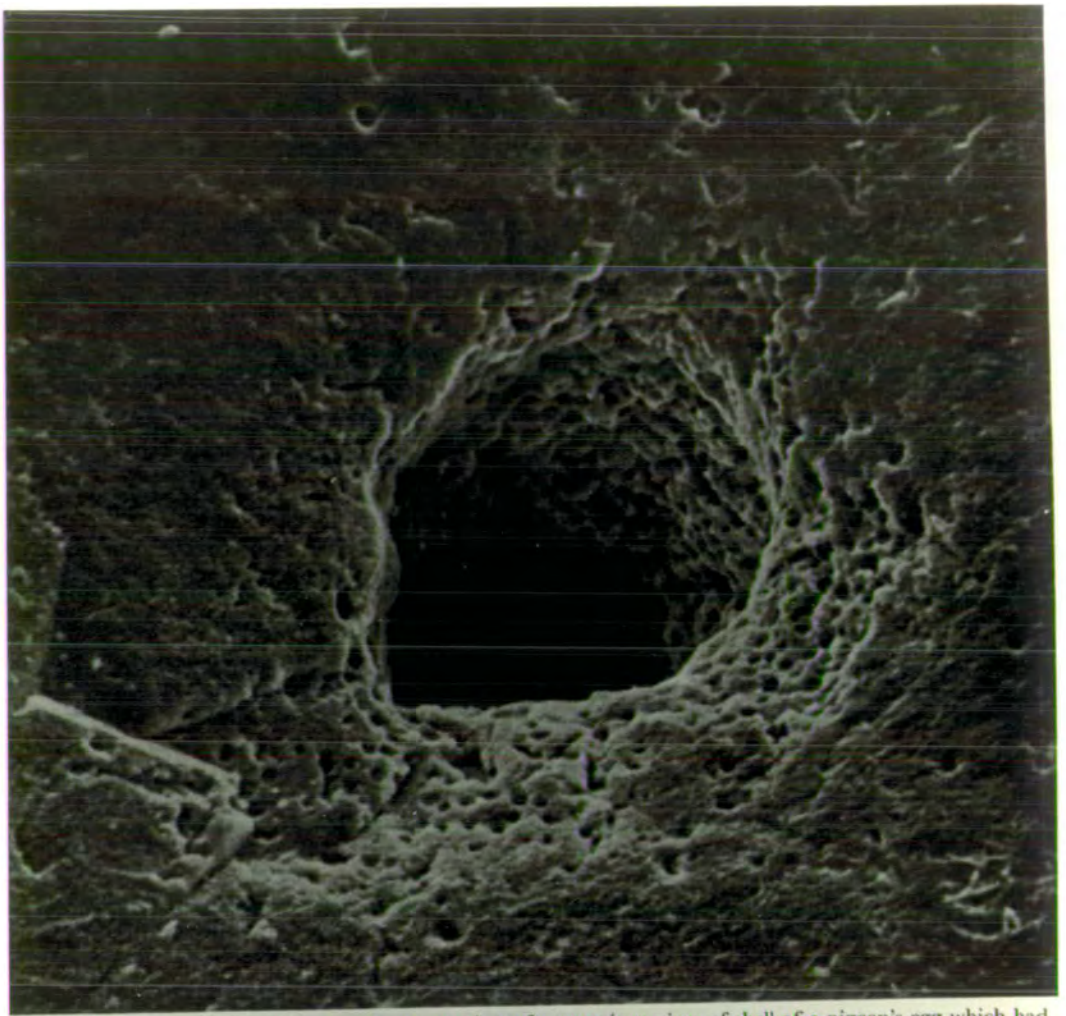


PLATE-FIG. 3.—Scanning electron microscope view of a pore in a piece of shell of a pigeon's egg which had been scoured for 5 min in boiling NaOH (5%, w/v). Beam angle, 45°; Mag, 1020; KV, 10.

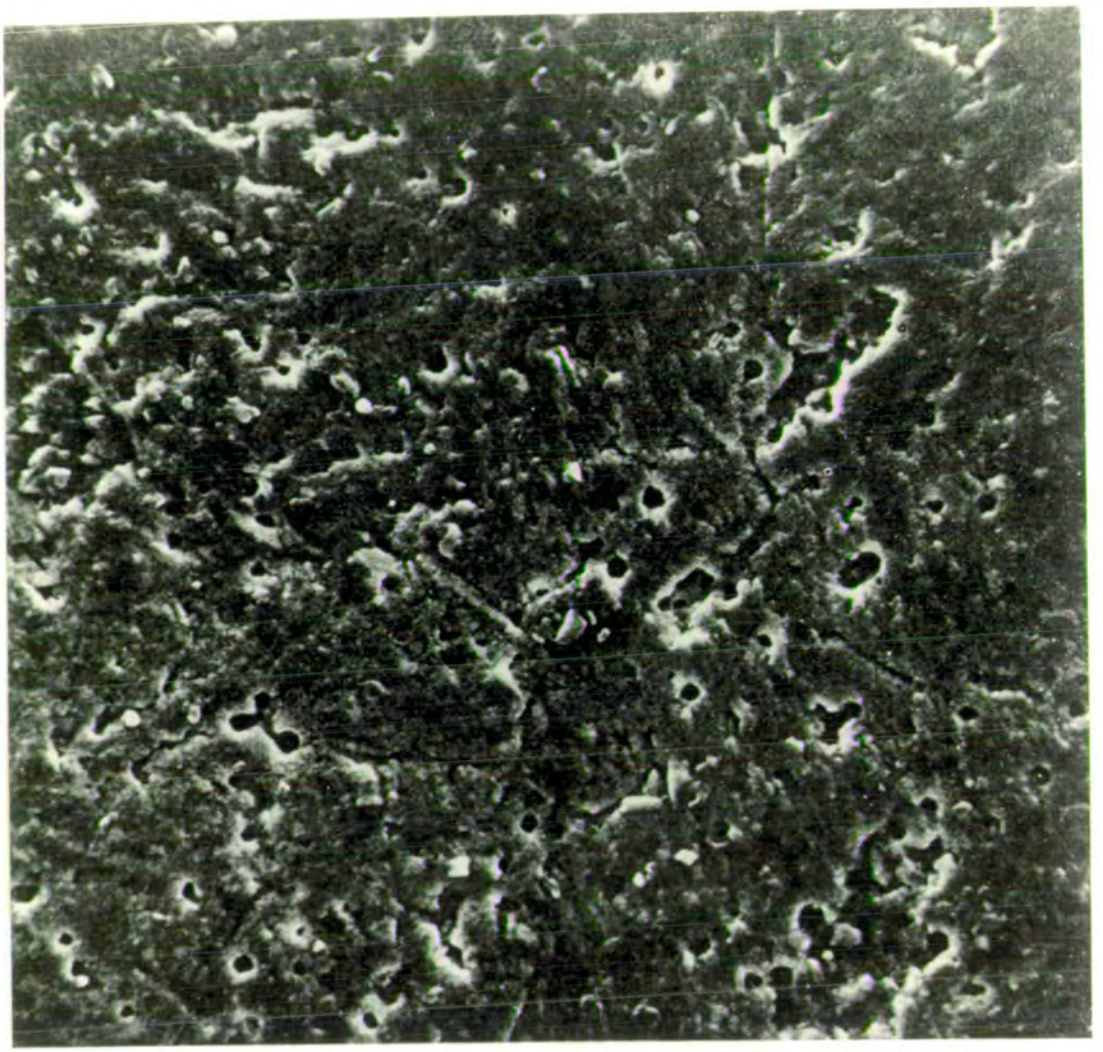


PLATE FIG. 4. Scanning electron microscope view of the outer surface of the shell from a pigeon's egg. Beam angle, 45°; Mag. 1140×; KV, 10.

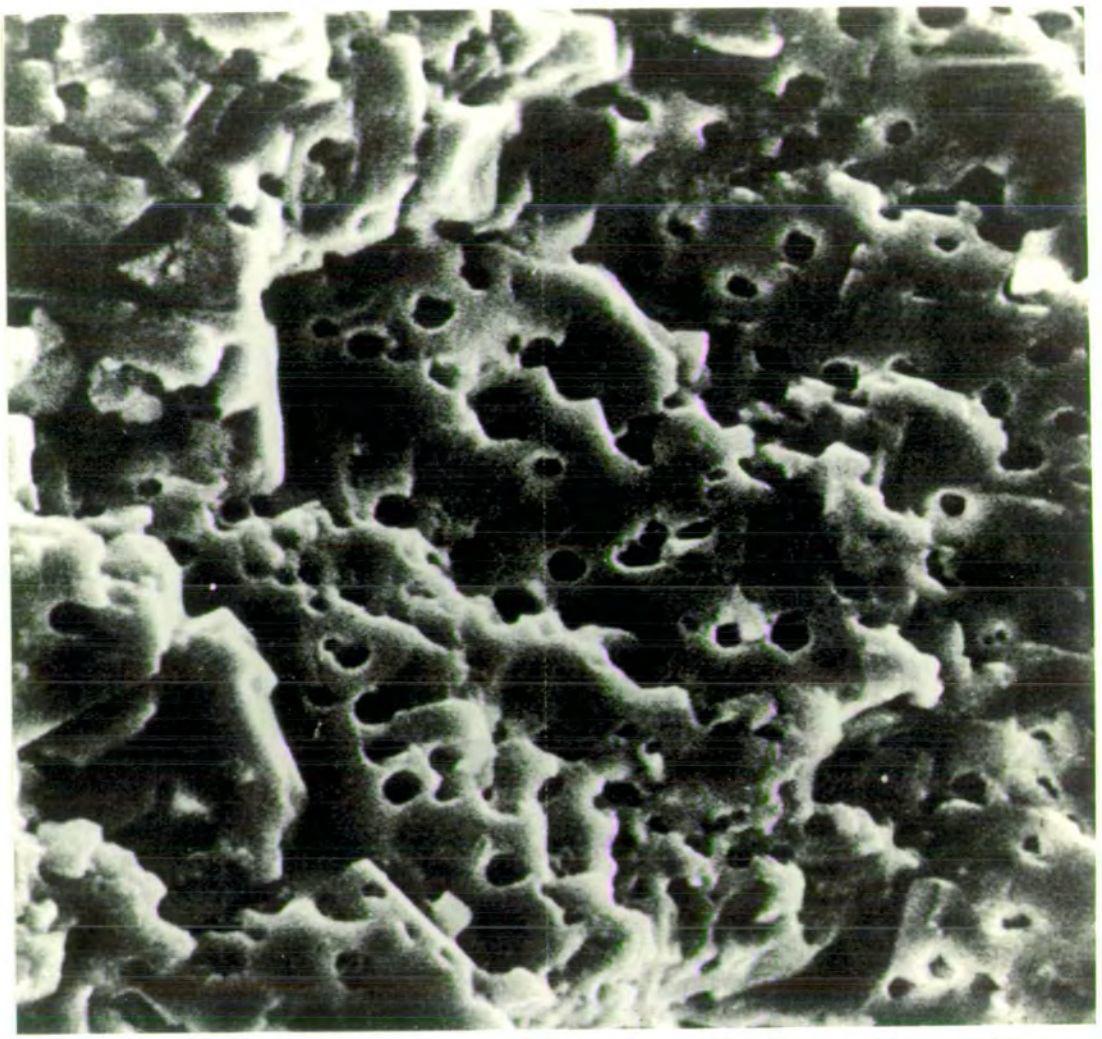


PLATE-FIG. 5.—Scanning electron microscope view of a small part of the face exposed by a radial fracture of the shell of a pigeon's egg. Beam angle 45 : Mag. 2700: KV. 10.

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THE CUTICLE: A BARRIER TO LIQUID AND PARTICLE PENETRATION OF THE SHELL OF THE HEN'S EGG

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SYNOPSIS

The cuticle of the egg shell prevents water-soluble dyes and carbon black from entering the majority of pores. A small percentage of eggs obtained from a commercial flock had no cuticle and their shells were easily invaded by carbon black. Chemical or physical removal of cuticle resulted in the pores being flooded with water which carried in carbon black. Such eggs did not, however, absorb water at a rate equal to that of eggs from which a piece of shell had been removed. This indicated that the pores without a cap and plug of cuticular material resisted the movement of water. The role of the cuticle and shell in repelling water is discussed and a function akin to that of the plastron of insect eggs proposed.

INTRODUCTION

Micro-organisms of extragenital origin are the principal cause for rotting of eggs (Board, 1968). They have to penetrate the integument of the egg before they can cause gross chemical changes of the white and yolk (Board, 1969). Of the major components of the integument, studies *in vitro* have shown that the inner shell membrane impedes bacterial movement more effectively than the outer shell membrane: the shell is of intermediate efficiency (Lifshitz *et al.*, 1964). Following microbial infection of the shell membranes of whole eggs there is a lag of 15 to 20 d before the onset of a generalised infection of the yolk and white (Board, 1966). This has been attributed (Board, 1964; Seviour and Board, 1972) to the failure of the initial contaminants to colonise the white rather than to their confinement by the shell membranes. It has been shown, for example, that organisms can be recovered from the inner surface of the shell membranes shortly after the surface of the shell has been challenged (Bean and McLaury, 1959; Williams *et al.*, 1968). Thus, from a practical viewpoint, the barrier provided by the shell to microbial penetration can be considered to be more important than that offered by the shell membranes.

With moulds (Weston and Halnan, 1927; Rosser *et al.*, 1942; Lorah *et al.*, 1954) or filamenteus bacteria (Cattaneo, 1877), the pores of the shell are penetrated by hyphae or filaments which have their origin in colonies growing on the outer surface of the shells of eggs stored at relative humidities greater than 90% (Sharp and Stewart, 1936). Although rot-producing bacteria can be deposited on the

shell membranes by these infiltrating filaments (Zagaevsky and Lutikova, 1944), they appear, in most instances, to be translocated (*i.e.* passively borne) along the pores when the latter are flooded (Haines and Moran, 1941). Some workers (*e.g.* Romanoff, 1931) have asserted that the cuticle plays a cardinal role in minimising bacterial penetration of the pores; others (Vadehra *et al.*, 1970*a*) have drawn the same conclusion from data which may be open to alternative interpretations (see Discussion), whilst others (Walden *et al.*, 1956; Kraft *et al.*, 1958; Brown *et al.*, 1965) have assumed that the " porosity " of the shell determines the extent to which the contents of eggs are contaminated. As will be seen in the Discussion, the term " porosity " has acquired a nebulous meaning partly because of the methods used to determine it and partly because of its use in unrelated contexts.

When reviewing the many studies of bacterial penetration of the shell of the hen's egg (Williams and Whitemore, 1967), it was obvious that the work had been rendered cumbersome by the need for asepsis, sterilisation, incubation, etc. Circumvention of these difficulties was suggested by the observation that, with the method of Board and Board (1967), *Serratia marcescens*, carbon black and water-soluble dyes were drawn into the same pores when eggs contracted in chilled water. This report gives the results of a preliminary study which sought to identify factors associated with the movement of both liquids and particles through the shell of the hen's egg.

MATERIALS AND METHODS

Staining the cuticle

The eggs were immersed in a 1% (w/v) aqueous solution of Edicol Supra Pea Green H (ICI, Hexagon House, Blackley, Manchester), and then rinsed with running tap water. The surface of the shell was scored for the intensity of the staining.

Eggs

These came from unmated flocks (housed in batteries) of commercial laying stock, one flock of white egg layers and another of brown egg layers. The eggs were obtained as nest clean and they were 3 or 4 d old when examined.

Bacterial penetration of the shell

The method of Board and Board (1967) was used.

Particle penetration of the shell

A 0·1% (v/v) suspension of carbon black vs paste in water was used. The particles were drawn into the pores by causing warm eggs to contract in a chilled suspension of the carbon black or they were forced in by imposing a pressure greater than atmospheric or by suddenly releasing a vacuum. The actual details varied with the experiments and they are given in the legends to the Figures.

Removal of cuticle

The cuticle was removed by immersing for 15 min in either a 10% (w/v) alkaline (pH 7·5) solution of ethylene diamine tetraacetic acid (EDTA), a 10% (w/v) NaOH or a 30% (w/v) solution of $Na_2S.9H_2O$. The eggs were held under running tap water and rubbed with paper tissues.

RESULTS

Sixty warm eggs (held at 39 °C for 1 h) from a white egg strain of a commercial laying stock were immersed in slush ice containing carbon black and *Serratia marcescens*. Thereafter, the eggs were treated as described by Board and Board (1967). After incubation at 30 °C for about 30 h the shells were bisected longitudinally. A spot of carbon black occurred at the centre of every patch of formozan which had been deposited in the shell membranes by bacterial reduction of tetrazolium. Although carbon black contains particles both larger and smaller than bacteria, this observation indicated that, in spite of the range in size, the particles penetrated only those pores which permitted the passage of bacteria.

Staining of the cuticle

The success which attends the apparently arbitrary choice of pigments by those who decorate eggs for festive occasions (Newall, 1971) suggest that the outer surface of the hen's egg has a low specificity for stains. It was found in a limited survey that a range of common laboratory dyes stained the shell surface, a finding that is in accord with those of Sharp (1932). For routine purposes, Edicol pea green was chosen because it provided a background in the membranes against which carbon black could be detected.

If eggs were immersed for 15 min in either a solution (10% w/v) of EDTA, 10% (w/v) NaOH or 30% (w/v) $Na_2S.9H_2O$ and then rubbed with paper tissues under running tap water the surface of the shell would not stain. Rubbing the shell with carborundum paper, either wet or dry, resulted in an abraded area which did not stain as deeply as the untreated parts of the shell. It was not, however, as free from cuticle as shells which had been cleaned chemically. The margin of the abraded area was deeply scored and the centre part was spotty presumably due to material which, through being in the orifice of the pores, had been protected from the abrasive. Conclusive evidence that the pigments were staining the cuticle came from the observation that when stained eggs were immersed in neutralised EDTA the stained cuticle came away like a veil leaving an unstained shell. This was most marked when brown eggs were used.

Role of solvents

Aqueous solutions of dyes did not penetrate untreated shells when the eggs and liquid were at the same temperature; limited penetration occurred when the shell was freed of cuticle (Fig. 7). A survey of solvents showed that rapid and extensive penetration of untreated shells occurred only with those having surface

tensions of less than 30 dyn/cm (Table 1). Two distinct situations obtained following the penetration of the shell. Dyes in water-soluble solvents (*e.g.* methanol and ethanol) formed discrete spots in the shell membranes. Water immiscible solutions of dyes spread rapidly over the cone layer of the shell giving smudged patches of pigment which did not appear to invade the membranes. The application of

TABLE 1

The role of surface tension in liquid penetration of the shell of the hen's egg

Surface tension[1] (dyn/cm)	Solvent	Dye	Appearance of treated eggs[2]: Surface of shell	Pores	Cone layer of shell	Shell membranes
< 30	Water	Edicol pea green	S	(p)	NS	DS
	Water/ethanol	Methylene blue	S	(p)	NS	DS
	Water/methanol	Methylene blue	S	(p)	NS	DS
> 30	Ethanol	Methylene blue	S	P	NS	DS
	Ethanol + water	Methylene blue	S	P	NS	DS
	Methanol	Methylene blue	S	P	NS	DS
	Methanol + water	Methylene blue	S	P	NS	DS
	Acetic acid	Waxoline blue	s	P	NS	DS
	Propyl alcohol	Waxoline blue	s	P	NS	DS
	Butanol	Methylene blue	S	P	NS	DS
	Amyl alcohol	Waxoline blue	s	P	MP	MP
	Xylene	Waxoline blue	s	P	MP	MP
	Benzene	Waxoline blue	s	P	MP	MP
	Chloroform	Waxoline blue	s	P	MP	MP
	Carbon tetrachloride	Waxoline blue	s	P	MP	MP

[1] As the actual surface tension of the solvents was not checked, the value of 30 dyn/cm has been arbitrarily chosen as a dividing point.

[2] The whole egg was dipped in a solution or the stain applied with a saturated piece of cotton wool to either a whole egg or a piece of shell. S, deeply stained; s, lightly stained; NS, not stained; (p) a few pores penetrated; P, many pores penetrated; DS, discrete spots of pigments and MP, smudged patches of pigment between the shell and shell membranes.

[3] Solvents given in **bold** lettering are water immiscible.

drops of dye solution to the shoulder of eggs standing on their pointed ends showed that wetting, as indicated by the drop running down and spreading out did not occur unless the solvent had a surface tension of less than 30 dyn/cm. Duck eggs were less easily wetted than hen eggs. This is to be expected from the many reports that their shells are water repellant (Romanoff and Romanoff, 1949; Lack, 1968).

Cuticle-less eggs

Of the 453 brown eggs examined 3·5% did not have any demonstrable cuticle while 8·0% had no cuticle at the pointed or broad pole. Eggs having no cuticle at the equator were not found. It is considered that these results reflect the situation obtaining at oviposition. Many of the eggs had scored cuticles, caused, presumably, by the hen's treading on the eggs or the eggs being scraped as they slid down the inclined wire mesh at the bottom of the battery cages. The eggs used in this study were nest clean. The farmer cleaned lightly-soiled eggs with a damp cloth but

this would not be expected to damage the cuticle in the light of our observations that extensive abrasion of the shell is required before its surface is rendered unstainable.

Penetration of pigments and carbon black

Pigmented spots with black centres were seen in the shell membranes of eggs which had contracted in a suspension of carbon black in Edicol pea green. Of the white eggs examined (Fig. 1), 48% had from 0 to 19 spots on the membranes,

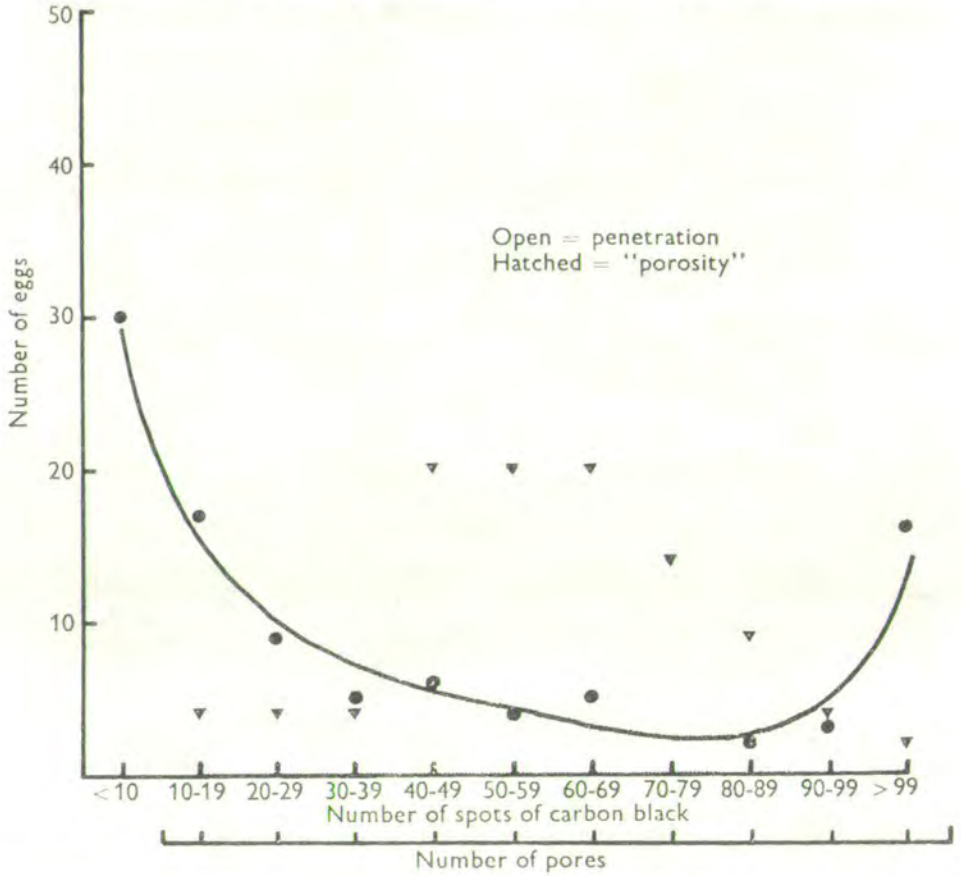

FIG. 1.—Points of carbon black penetration of the shells of white eggs. Eggs were kept for 1 h at 39 °C and then immersed for 15 min in slush ice containing carbon black (0·01% w/v) and Edicol pea green (0·1% w/v). Number of eggs, 97. Penetration with carbon black (●); pores/piece of shell, 9 mm diameter (▲).

35% had from 20 to 99 but only 17% had more than 100 spots. The spots did not appear to be congregated at one pole of the egg. In a study of 60 eggs, 20 had more pores in the blunt than the pointed ends, 20 gave the contrary pattern and the other 20 had equal numbers of pores in the two halves of the shell.

The white eggs had rough shells and the penetration of carbon black was often associated with calcareous lumps on the shell. In their investigations of

antibiotic absorption by eggs Alls *et al.* (1963, 1964) noted that the antibiotic penetrated through " sandy " areas or in the region of small lumps on the shell. They concluded that such areas and excrescences would not be covered with cuticle. Although in our experiments deformations were associated with many of the pigmented spots on the shell membranes of white eggs, they did not account for the difference in the distribution of the number of points of pigment penetration of the white and brown eggs.

TABLE 2

Microscopical examination of shells of hen's eggs

Staining	No. stained patches[1]	No. of pores[1] in: Untreated shell (light transmitted through pore)	After treatment with NaOH: light transmission	After treatment with NaOH: methylene blue	After acid etching
Heavy (Brown eggs)	31·3[2]	0	t	ND	7·0
	37·6	0·2	t	ND	7·2
	38·4	0	t	6·4	6·2
	34·4	0	t	6·2	7·2
	37·4	0	t	6·2	6·4
	45·8	0·2	t	7·4	7·0
	30	0·1	t	9·8	9·0
	30	0·1	t	8·4	10·6
	30	0·1	t	8·2	11·0
	30	0·1	t	10·2	11·8
Light (White eggs)	13·6	0·2	1·4	6·9	7·8
	8·3	0·4	3·2	3·8	B
	7·5	1·0	2·3	6·6	7·7
	5·5	0·6	2·5	5·1	5·3
	7·0	0·1	1·7	4·5	5·1
	7·0	6·5	0·7	7·2	7·6
Absent (Cuticle-less brown eggs)	—	6·6	7·6	7·6	7·0
	—	5·0	5·6	5·0	6·4
	—	4·3	6·4	5·8	6·2
	—	5·7	5·0	5·4	7·0

—, None; B, shell broken; t, none seen; ND, not determined.

[1] Per microscopic field.

[2] Average of 5 readings.

The white eggs did not stain as deeply with the pea green dye as did the brown eggs. Microscopic examination (either with transmitted or reflected light) showed that the stain tended to occur in patches which were irregularly fissured. Romankewitsch (1934) noted a granular structure in the cuticle and that there were fewer granules in the cuticle on white eggs. In our experiments the incidence of pigmented patches appeared to be proportional to the quality of the cuticle. Thus the cuticle of brown eggs (i.e. those having a high incidence of patches, Table 2) was removed as a veil during EDTA treatment whereas that on white shells was not easily removed and then only as flakes. During microscopical examination, pores which transmitted light were seen in white and cuticle-less eggs but rarely in brown eggs (Table 2). Moreover, there was no correlation

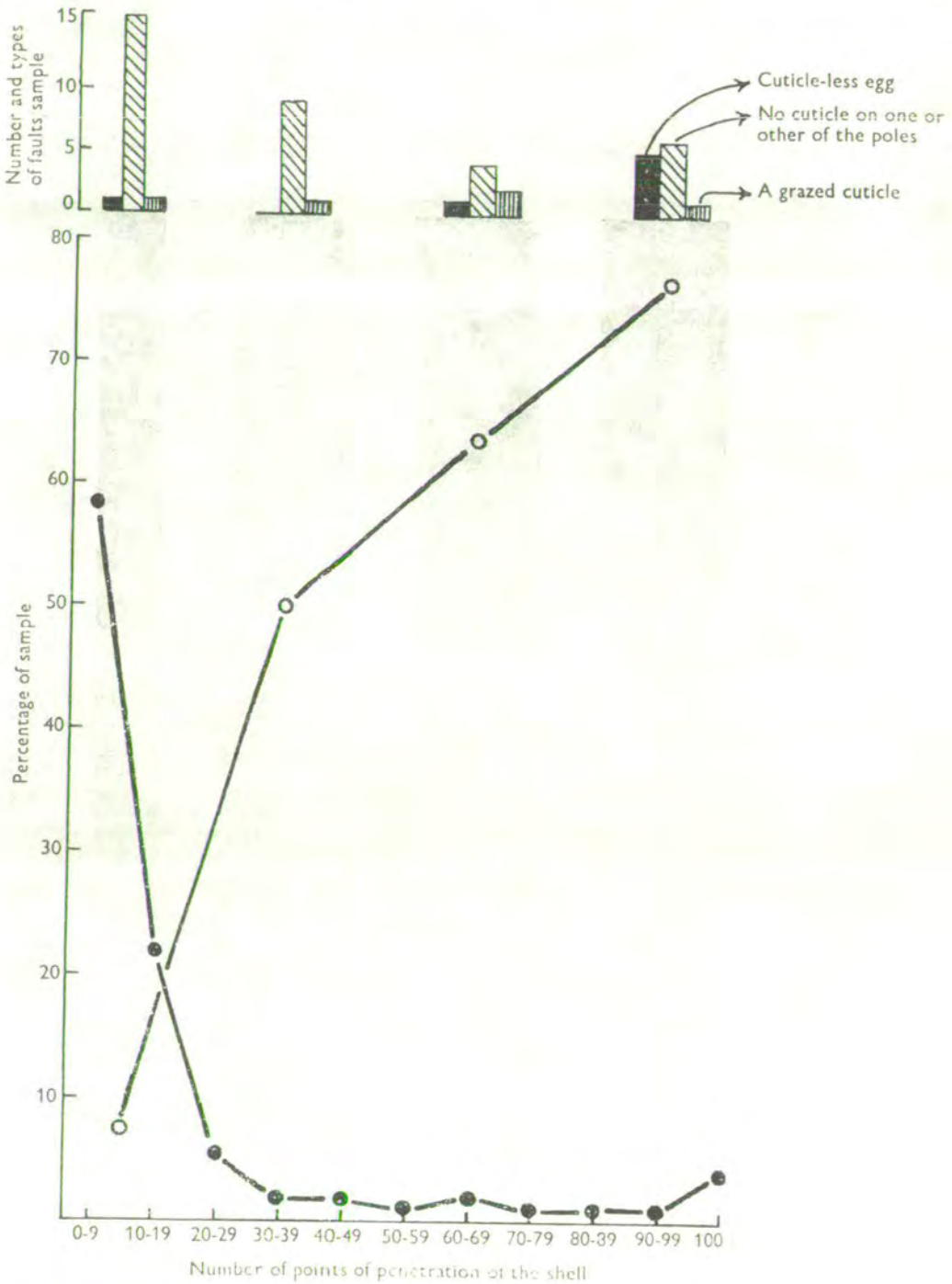

FIG. 2.—Points of carbon black penetration of the shells of brown eggs. Eggs were kept for 1 hr at 30° C and then immersed for 15 min in slush ice containing carbon black (0·01% w/v) and Edicol pea green (0·1% w/v). Number of eggs, 195. Points of carbon black penetration (●); percentage of faults (○) in the sample included in the shaded area, and histogram type of faults present in the sample included in the shaded area.

between the incidence of the irregular stained patches and the shells' content of pores (Table 2), thus the patches noted in this study are not the same structures as those which Marshall and Cruickshank (1938) referred to as "plaques". Keeping white shells in boiling NaOH (2·5% w/v) for 15 min increased the incidence of pores which transmitted light but their number was less than those which could be stained with methylene blue and the latter count was of the same order (Fig. 3) as the pore counts obtained with shells which had been etched (Tyler, 1953) with nitric acid. Treating cuticle-less eggs with NaOH resulted in only a slight increase

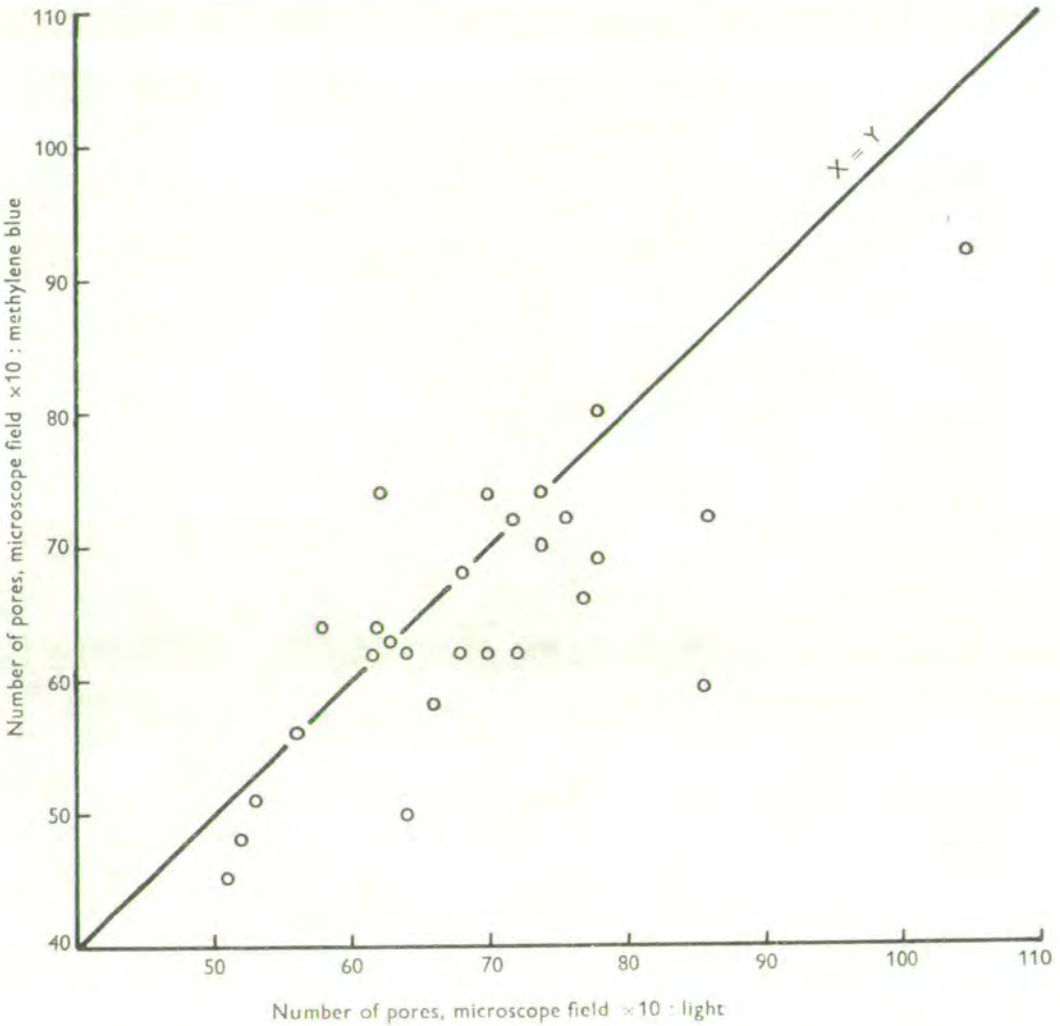

FIG. 3.—Comparison of methylene blue and light transmission for enumeration of pores. Shells were boiled for 15 min in 2·5% (w/v) NaOH, then painted, on inner surface, with a 1·0% (w/v) ethanolic solution of methylene blue and number of coloured spots in outer surface counted when shell viewed by transmitted light on microscope. The same piece of shell was etched for 25 sec with nitric acid and the number of pores transmitting light enumerated when shell viewed with transmitted light under microscope. Each point, the mean of 5 readings taken from randomly selected microscope field.

in the numbers of pores which transmitted light (Table 2). With brown eggs having a high incidence of coloured patches, NaOH did not scour the pores sufficiently to permit light transmission. These observations indicate that, in addition to capping the outer orifice of the pores, the cuticle may also be involved in plugging the pore canals. Thus the differences in the scatter in Figures 1 and 2 may be accounted for in part by the poor amount of the cuticle on the white eggs providing a less efficient covering and plugging of the pores.

When the brown eggs having large numbers of points of penetration of carbon black were examined (Fig. 2) it was noted that a large percentage of the eggs had either no cuticle or a damaged one. Such eggs were present, but as a low percentage only, in the group of eggs which had from 0 to 19 points of carbon black penetration. This was taken to indicate that, although some eggs did not stain with Edicol pea green, there had been deposition of cuticle in the pore canals sufficient to impede the movement of carbon black. This analysis did not, however, identify the situation obtaining in brown eggs which had heavily stained cuticle but a high incidence of penetration of carbon black. In the absence of demonstrable damage to the cuticle, this may indicate shells in which there were minor faults in the deposition of the cuticle.

As cleaning the shells with NaOH did not free penetrated pores of carbon black, it was possible to recognise them in models of the shell prepared by the methacrylate method of Tyler (1956). Because of uncertainty about the extent of contraction during the polymerisation of methacrylate, measurement of the pore models was not attempted. Instead the moulds were broken after decalcification so that the plastic was severed at the mammillary face and the mould viewed under an epi-technoscope. Black columns of plastic which were thicker than the moulds of the majority of pores were occasionally found. Nevertheless, carbon black was present in many of the casts of average size. Some pores, especially those of above average diameter, had a wide and deeply recessed outer orifice but such large orifices were not invariably contaminated with carbon black. These observations suggest that shells contain a few malformed pores which permit the easy access of carbon black. Nevertheless, the presence of carbon black in pores of average size indicates that malformation of pores could not account entirely for the ingress of particles into the shell and it was the efficiency of covering and plugging of the pores with cuticle which was of primary importance. In the reports of Rauch (1952, 1954), there was an accentuated tail in the scatter of his observations and he accounted for this in terms of pore diameter. It could presumably be accounted for in terms of the extent of penetration of the pores with NaCl; extensive penetration could be expected to increase a shell's conductivity without a concomitant requirement for a high incidence of pores having large diameters. In the present study (Fig. 1) the number of pores in the shells appeared to be normally distributed and no success attended attempts to correlate the incidence of carbon black penetration and the porosity of the shell.

The importance of the cuticle in impeding particle penetration of the shell was emphasised (Plate-Fig. 1) by eggs which had the cuticle removed from half the shell before being challenged with carbon black suspended in Edicol pea green (Fig. 4). The incidence of penetration was greatly increased when EDTA or NaOH but to a lesser extent when Na_2S or carborundum were used to remove the cuticle. With

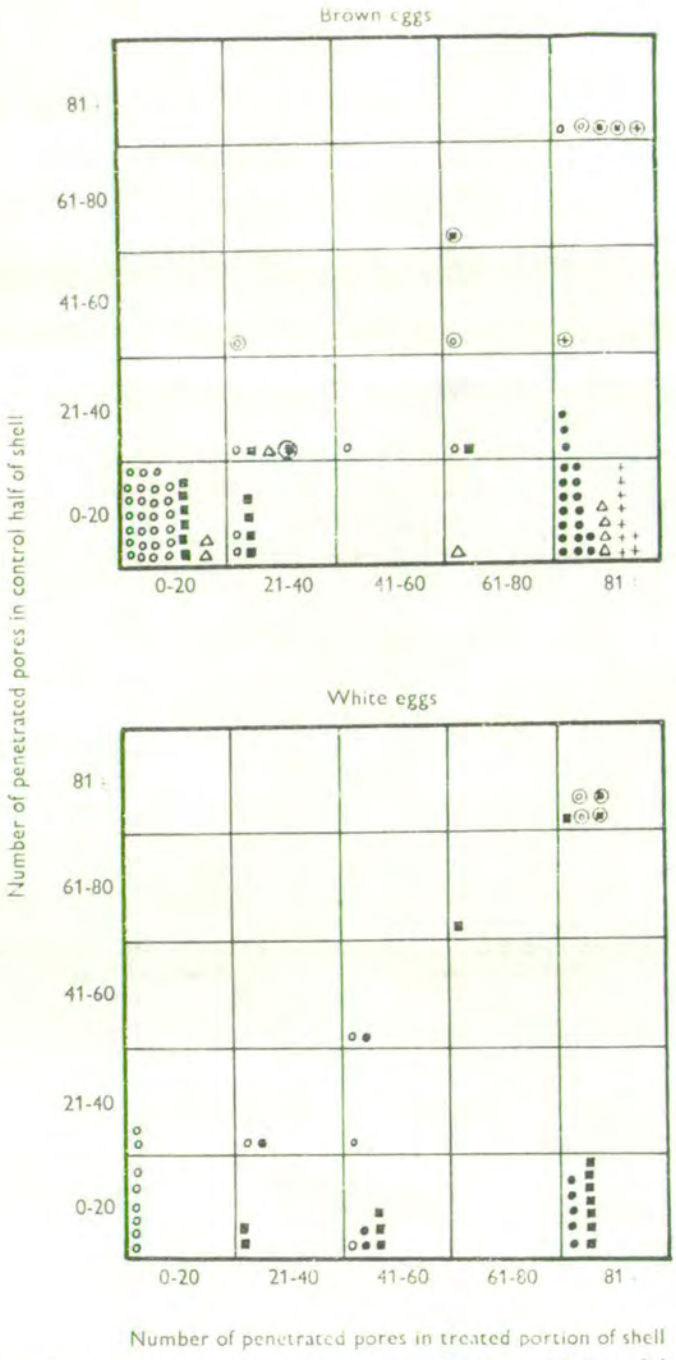

FIG. 4.—Comparison of carbon black penetration of treated and untreated halves of the same shell. Treatments: ○ = half of shell immersed for 15 min in water; ● = immersed for 15 min in 5% (w/v) EDTA (pH 7·5) and rubbed with paper tissue; + = immersed for 15 min in 10% (w/v) NaOH; △ = immersed for 15 min in 30% (w/v) $Na_2S.9H_2O$; ■ = rubbed with wet carborundum. Circled symbols refer to eggs having no cuticle at oviposition.

After treatment the eggs were kept at 39 °C for 1 h and then immersed for 15 min in slush ice containing carbon black (0·01% w/v) and Edicol pea green (0·1% w/v). The eggs were opened and number of pigmented spots on shell membranes enumerated.

the latter method, the increase with white eggs was greater than that for brown ones but this may merely reflect the vigour with which the operator rubbed the shell. It was notable that the results obtained with untreated eggs which were cuticle-less at oviposition tended to be scattered (Figure 4) between those of the control and treated eggs. The incidence of penetration was not affected by keeping eggs in a detergent (Teepol) thus suggesting that the surface energy of the cuticle was not reduced by the absorption of a surfactant. Similarly, treating eggs with a drying agent such as methanol did not increase penetration. This was taken to indicate that dehydration of the cuticle did not cause significant contraction of the plug within the pore canal. Our observations that the removal of cuticle enhances liquid penetration of the shell of the hen's egg is in accord with those of Alls *et al.* (1964) who obtained similar results with eggs which had been treated with hydrochloric acid, a process which may also of course erode the pore canals.

It has been often noted (Weston and Halnan, 1927; Rosser *et al.*, 1942; Lorah *et al.*, 1954) that storage in a humid atmosphere leads to extensive mould growth on the shell surface. In the present experiments, the growth of streptomyces was associated with the digestion of the cuticle and extensive penetration when eggs were immersed in a chilled suspension of carbon black (Plate-Fig. 2).

Water uptake

The immersion of warm, untreated eggs in iced water did not result in all eggs increasing in weight (Fig. 5). The majority changed by about 100 mg, a range similar to that obtained when untreated eggs were kept for 15 min in water at the same temperature (Fig. 7). The number of points of penetration of chilled eggs with pea green dye is shown in Figure 5. It was noted that 80% of the eggs fell in the cells covering the events: weight change, about 100 mg × points of penetration, 1 to 20. Of the 52 eggs whose shell membranes contained no spots of dye, 29 had gained more than 100 mg. Of the 69 eggs having from 1 to 20 points of penetration, 32 had lost weight. These results indicate that the pores of the egg can be flooded without the egg actually absorbing sufficient water to give a demonstrable weight increase. It is noteworthy that Bryant and Sharp (1934) noted a loss in weight following the washing of eggs. At this time, no explanation can be offered to account for this. The results obtained for untreated eggs was markedly skewed away from the peak given by the majority of observations (see also Figs 7 and 8), a similarly skewed distribution of " pore size " was noted by Rauch (1952, 1954). Judging from the distribution of points of penetration of the untreated eggs (Fig. 5, inset) this tail in the distribution could not be accounted for in terms of the number of pores flooded. An analysis of the eggs showed (Fig. 6) that those which absorbed from 11 to 700 mg water had either broken shells, no cuticle or grazed cuticles. With eggs which had had the cuticle removed from about half the shell, there was a higher incidence of eggs which had gained from 110 to 250 mg water (Fig. 5). Nevertheless, some of the treated eggs showed a loss in weight and some an increase of more than 300 mg. A loss in weight was a feature when treated eggs were placed in water at the same temperature (Fig. 7). When the weight change occurring with chilled, treated eggs was plotted against points of penetration (Fig. 6), there was an apparent correlation between the

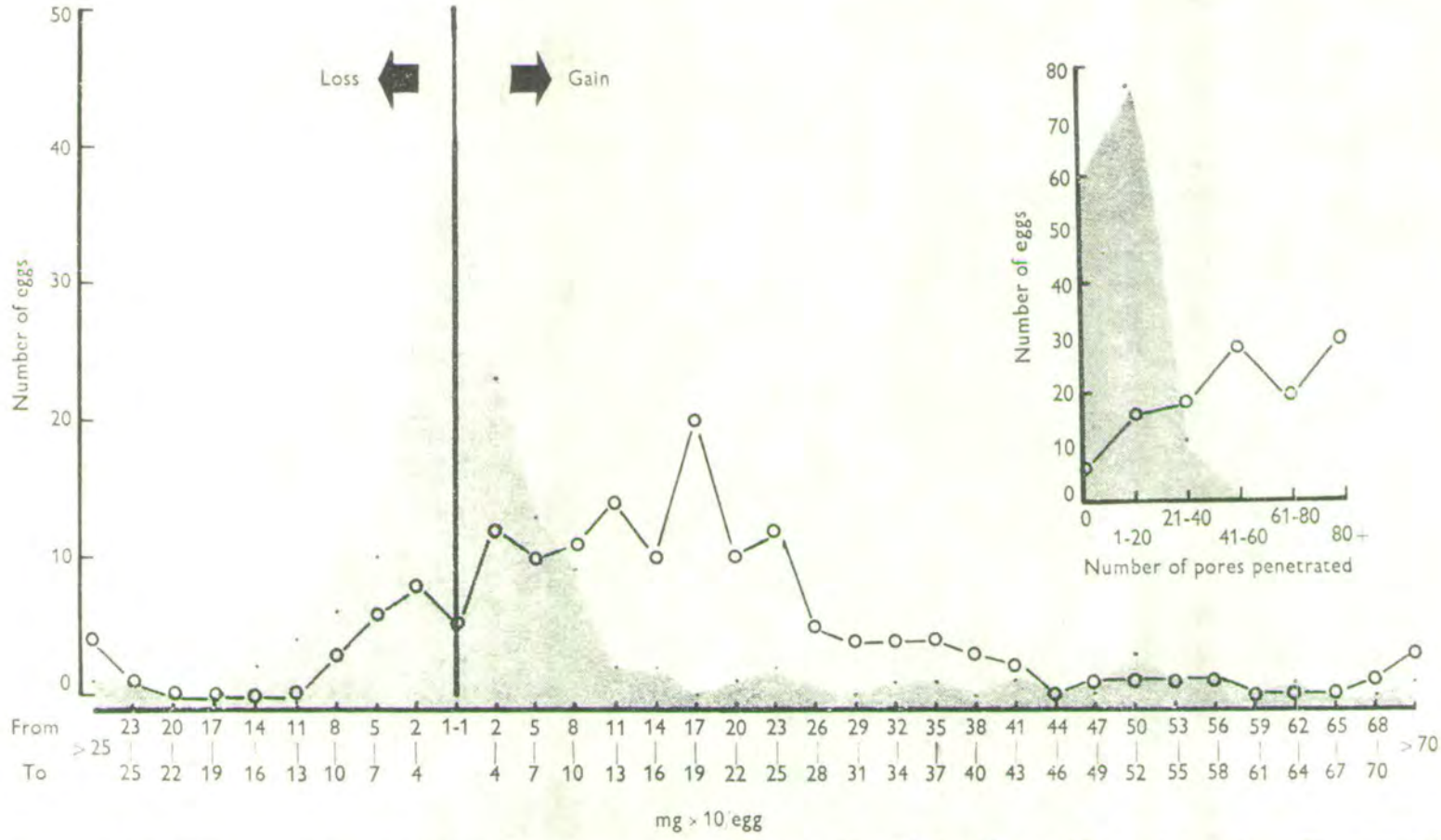

FIG. 5.—Weight change in eggs immersed in slush ice. Brown eggs were kept for 1 h at 39 °C, weighed and immersed for 15 min in slush ice. They were then dried and re-weighed. Stippled area, control eggs; ○ = eggs from which the cuticle from half the shell had been removed with EDTA. **inset**: number of pores penetrated in control (stipple) and EDTA-treated (○) eggs.

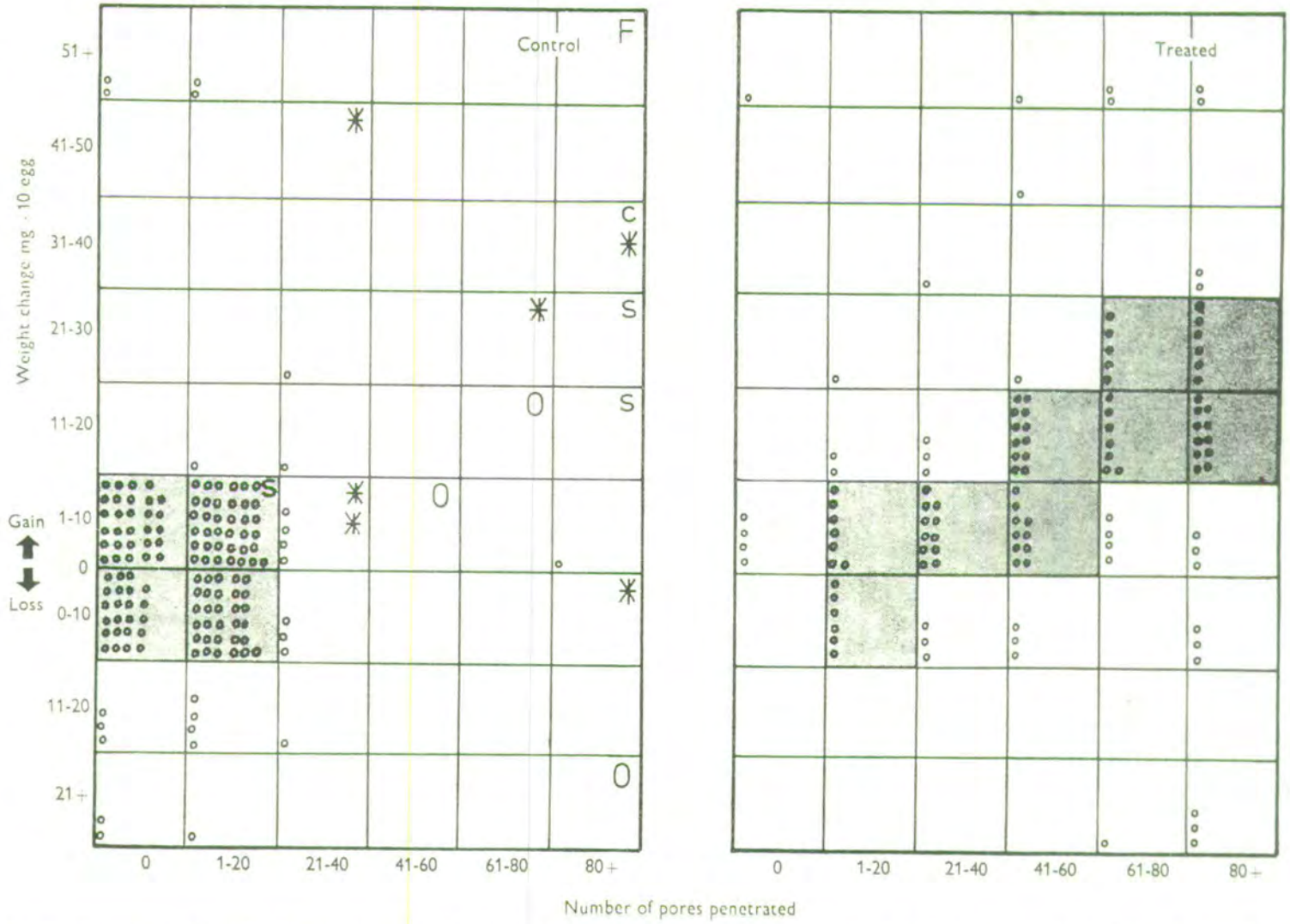

FIG. 6.—Comparison of weight change and points of penetration of shell with carbon black. Experimental details given in the legend to Fig. 5. The stippled areas represent 80% of observation with control eggs and 66% with treated eggs. Asterisk, no cuticle at pole; large open circle, no cuticle; S, scratched cuticle, and C, cracked egg.

amount of water absorbed and the number of points of penetration of an egg. A similar relationship was noted by Alls *et al.* (1964). These results indicate that the cuticle, by covering and blocking the pores, plays an important role in waterproofing the shell.

In the work discussed to date, the contraction of a warm egg in chilled water was used to promote flooding of the pores in the shell. Since the introduction of this technique (Haines and Moran, 1941), it has been generally assumed that flooding results from suction generated by the contraction of the white and yolk being greater than that of the shell and its membranes. If the pressure differential thus created was satisfied entirely by the absorption of water, then it could be expected that eggs would increase in weight by roughly the same amount. Such a hypothesis was not supported by the present study (Fig. 5). Could it be, therefore, that pressure differential was satisfied in part by the absorption of water and in part by the eggs taking up gases dissolved in the chilled water? It has been suggested (Board, 1966) that osmotic forces acting across the shell membranes would promote the movement of water along a flooded pore canal. The osmotic uptake of water by eggs having a part of their shell removed is illustrated in Fig. 7. Nevertheless, we have been unable to demonstrate this force acting in eggs having intact shells. Thus with eggs held in water under a positive pressure of 0·35 kg/cm^2, the amount of water absorbed by the eggs was similar to that absorbed by those which had been held in iced water (Fig. 7). A marked increase in the amount of water taken up by cuticle-less eggs (but not the control eggs) was achieved by creating and suddenly releasing a vacuum in the head space of a container having eggs immersed in water (Fig. 7). When eggs were held under negative pressure it was noted that a continuous stream of air bubbles came from 10 to 20 pores of untreated shells within seconds of suction being applied. This was also noted by Bryant and Sharp (1934) and these pores are presumably those which we consider are free from cuticle, either as a cap or a plug. As the vacuum increased, the surface of the egg became frothy presumably as a result of the air sucked from the majority of the pores being trapped by a canopy of cuticle over the orifice of the pores. With cuticle-less eggs, there was a continuous release of bubbles from the shell. When the vacuum was released, the eggs absorbed water (Fig. 7) and the membranes underlying the shells freed of cuticle were uniformly stained with dye. Thus, negative pressure had apparently drawn the air from the interface of the shell and its membranes rather than from the air space, the latter being considered by Voeten (1965) to be the origin of gas escaping through the shells of the eggs he subjected to vacuum.

Keeping eggs in water for more than 30 min following the release of vacuum did not increase the amount of water absorbed by them (Fig. 8) and there was no correlation between weight increase and number of pores penetrated (Fig. 9).

Water loss

There was no demonstrable difference in the rate or amount of water lost by control and cuticle-less eggs (Fig 10). The results obtained with the control eggs are similar to those of Pringle and Barrot (1937). Exposing a cuticle-less egg to an air current for more than 8 h did not lead to water losses different from

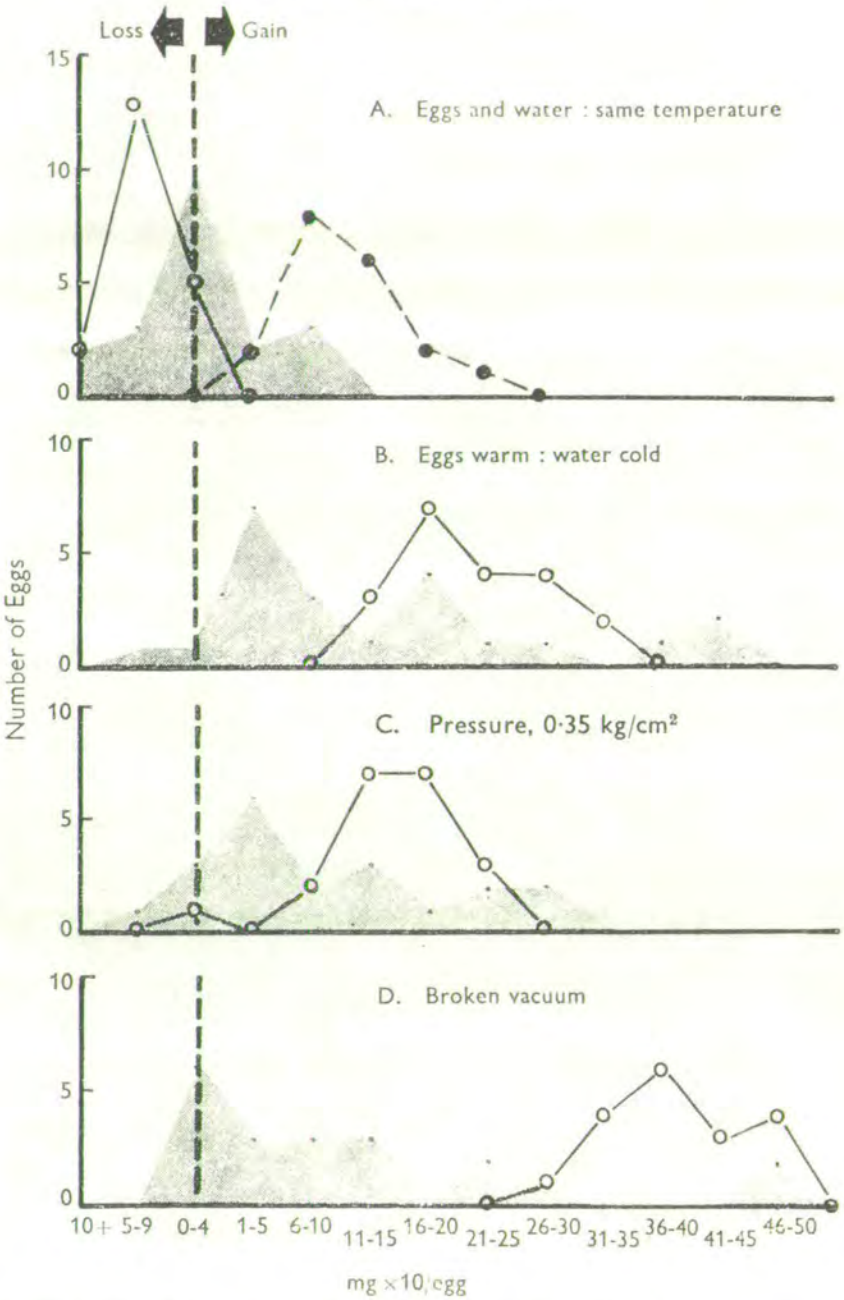

FIG. 7.—Weight change in eggs exposed to hydrostatic pressures generated in different ways. A: eggs and dye solution at same temperature; B: eggs kept for 1 h at 39 °C, then immersed for 15 min in slush ice containing carbon black and pea green; C: eggs submerged in dye solution (both at the same temperature) in a closed container and a pressure of 0·35 kg/cm² maintained by controlling the fine adjustment on a hydrogen cylinder; D: eggs immersed in a dye solution in a closed container were subjected to negative pressure until shells became "frothy" and then the vacuum released. Treatment period, 15 min. Control eggs (stippled areas); EDTA-treated shells (○); eggs from which a square (about 5 × 5 mm) of shell had been removed (●).

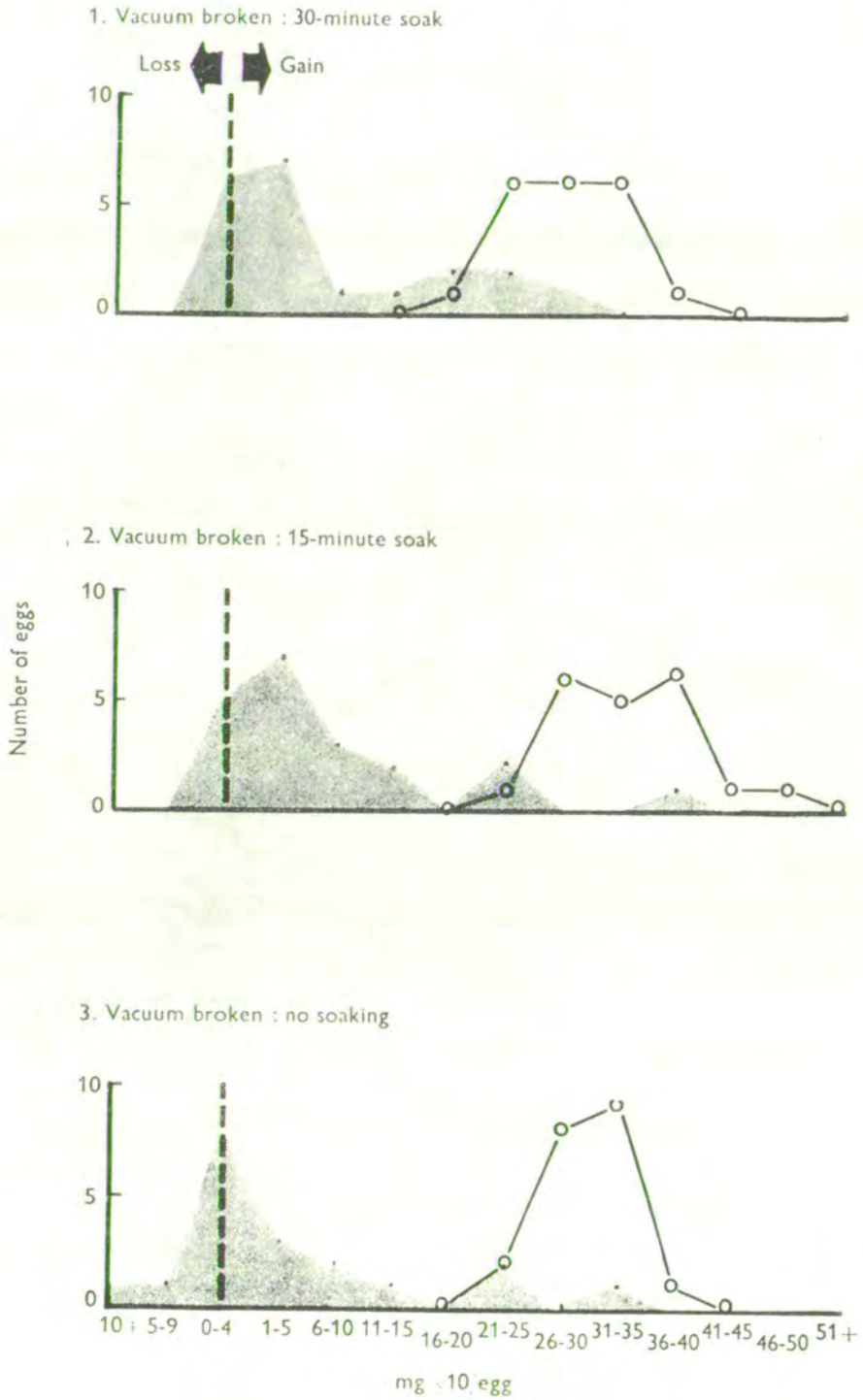

FIG. 8.—Weight change in eggs. Eggs were immersed in dye solution at same temperature and a vacuum drawn. At times indicated, eggs were dried and weighed. Control eggs (stippled areas); shells treated with EDTA (○).

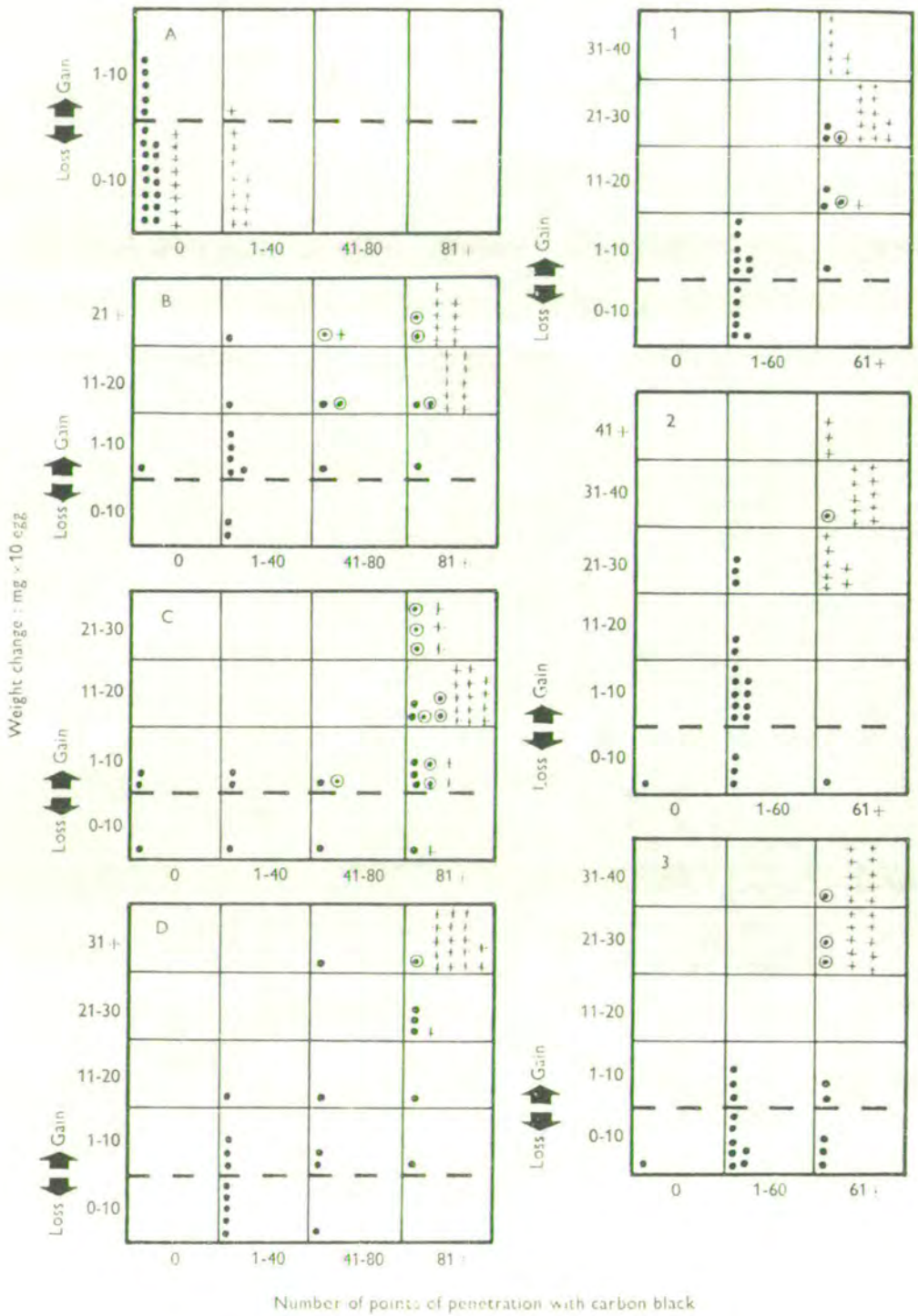

FIG. 9.—Comparison of weight change and points of penetration of the shell of eggs treated as described in Figures 7 and 8. A, B, C and D the same as in Figure 7 and 1, 2 and 3, the same as in Figure 8. Control eggs (●); egg shells treated with EDTA (+); encircled symbols refer to egg shells with damaged cuticles.

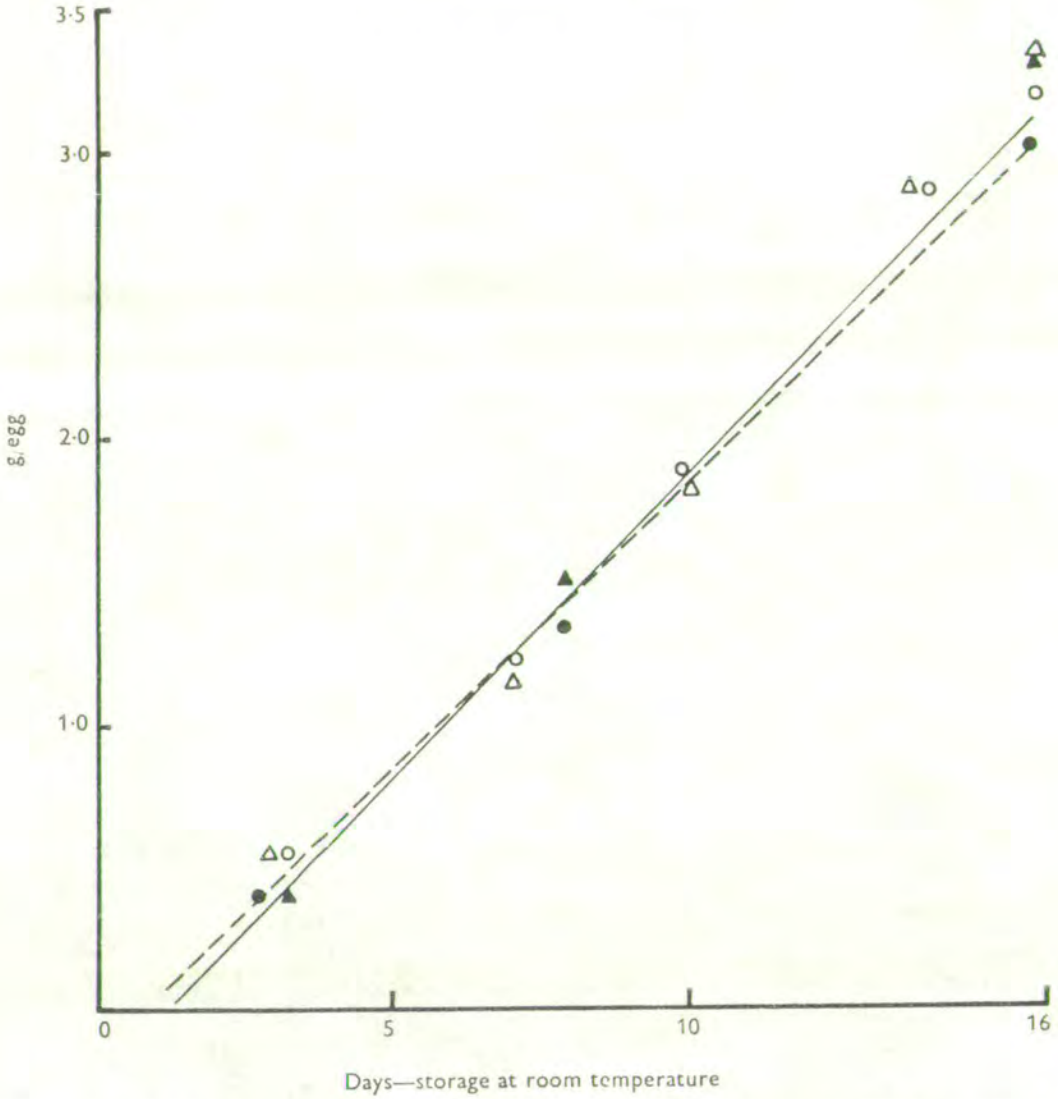

FIG. 10.—Weight loss by eggs stored in the laboratory. Open and closed triangles, EDTA treated and open and closed circles, control eggs.

those of control eggs kept under the same conditions. A difference in rate of water loss by control and cuticle-less eggs was noted, however, during storage under low humidities (Fig. 11), indicating that the cuticle may be involved in water conservation when eggs are exposed to dry conditions.

DISCUSSION

This study appears to be the first to identify some of the factors which contribute to the movement of liquids and particles across the shell of the hen's egg. For convenience, the practical implications are discussed under separate headings.

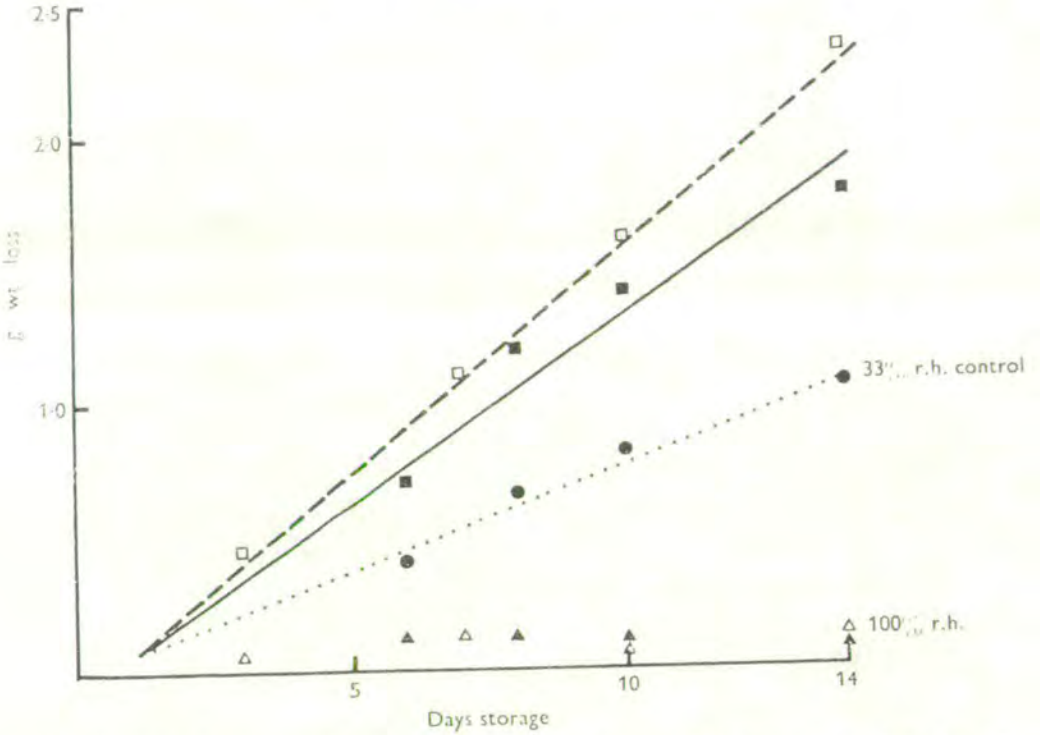

FIG. 11.—Weight loss by eggs stored in desiccators containing solutions which gave various relative humidities as indicated on Figure. EDTA-treated eggs at 7% r.h. (□); control eggs at 7% r.h. (■); control eggs at 33% r.h. (●); EDTA-treated eggs at 100% r.h. (△); control eggs at 100% r.h. (▲). Number of eggs per sample was 20.

Waterproofing

In nature, the embryo makes conflicting demands on the integument of the egg. Thus the shell has to contain sufficient pores for the exchange—not necessarily unimpeded (Romijn and Roos, 1938; Visschedijk, 1968)—of respiratory gases (6·6 g O_2 and 7·6 g CO_2 during the incubation of a 60 g egg; Romanoff, 1967) without being so weakened that it would be easily cracked or crushed. According to Tyler (1955, 1969*a*), their distribution (an arrangement lying somewhere between randomness and complete uniformity) provides an optimal density with minimal weakening of the shell of the hen's egg. Gaseous exchange must occur without a concomitant water loss from the embryo. It has been deduced (Gillespie and Scott, 1950) from the data of Smith (1934) that there is a logarithmic gradient of water vapour pressure along the pore canal and it is probable that this contributes to water conservation—in other words, water loss from eggs is not akin to evaporation from the surface of unglazed ceramic. The present study indicated that the cuticle (Fig. 11) contributes to water conservation only when eggs are stored at low humidities.

It would seen reasonable to assume that the shell must not become waterlogged otherwise the embryo would be asphyxiated. Some of the factors which may be

associated with the movement of liquids across the shell have been noted in Figs 12 and 13. It has been demonstrated repeatedly that work has to be done to overcome the surface energy (water repellancy) of the shell. In the laboratory,

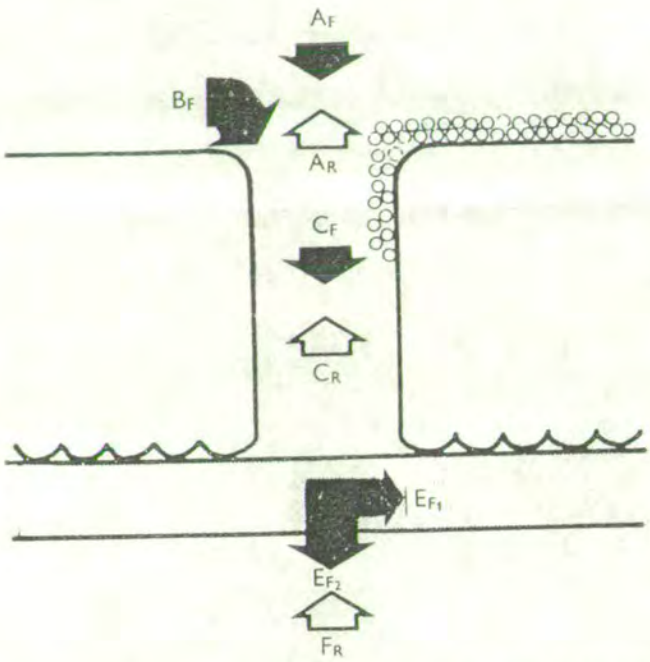

FIG. 12.—Proposed forces which influence the flooding of the shell of the hen's egg with water or other solvents having surface tensions greater than 30 dyn/cm. *Force*—A_F = hydrostatic pressure; B_F = wetting; C_F = capillarity; E_{F_1} and E_{F_2} = capillarity and osmosis. *Resistance*—A_R = cuticles covering and blocking of pore; C_R = pore canal architecture and plugging; F_R = back pressure due to air cell.

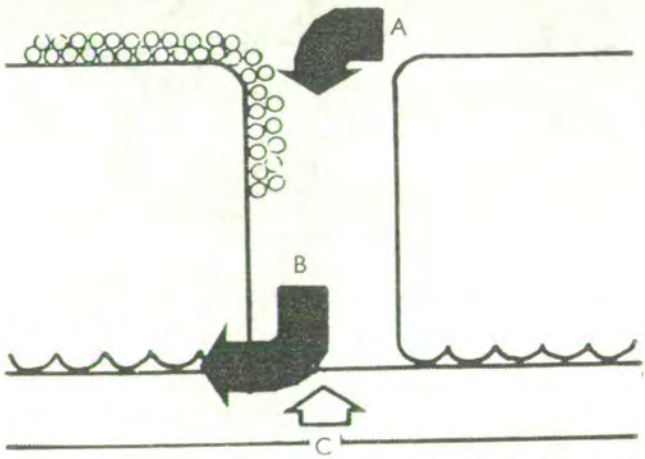

FIG. 13.—Proposed forces which influence the flooding of the shell of the hen's egg with solvents immiscible with water and having surface tensions of less than 30 dyn/cm. *Force*—A = wetting; B = capillarity. *Resistance*—C = immiscibility with water.

this is achieved by either causing a warm egg to contract in chilled water (Haines and Moran, 1941), applying a pressure to water in which eggs are immersed (Alls *et al.*, 1963), or releasing a vacuum in the head space of a vessel containing eggs immersed in water (Voeten, 1965). In nature, the first-mentioned would appear to be the most likely cause of flooding of the pore canals and it is in this situation that the cuticle makes a significant contribution to the water repellancy of the shell of the hen's egg (Fig. 1). Once this barrier has been overcome by hydrostatic pressure, other factors appear to impede the movement of water along the pore canal and into the space between the inner surface of the shell and its membranes.

The present study (Figs 7 and 8) showed that pores freed of the cuticular cap imposed a resistance (drag) to water movement greater than the pull generated by osmotic forces acting across the shell membranes, the latter being considered as a bundle of capillaries having holes of approximately 1 μm diameter (Wolken, 1951; Simons and Wiertz, 1963; Bellairs and Boyde, 1969). The drag may be due in part to the properties of the wall of the pore canal, in part to hydrostatic pressure forcing the cuticle into the pore canal (a suggestion based on the observations of Haines and Moran, 1937), and in part to the hydration and swelling of the mucin in the cuticle (Cooke and Balch, 1970*a*, *b*). The latter cause of drag is suggested by the work of Haines and Moran (1941). They noted that, with a constant pressure of 600 mmHg, the rate of movement of water through the shell decreased with time. Although osmotic forces may be initially negated by drag, they may subsequently contribute to drag by breaking the thread of water in a pore canal thereby introducing an air lock. In addition, osmotic forces appear to draw water from the inner orifice of the pore canal thus preventing the flooding of the spaces between the shell and its membranes. In this situation, the architecture of the cone layer of the shell may act as a hydrafuge. The observations made on shells treated with liquids having surface tensions of more than 30 dyn/cm indicate that the angle of the cone to the shell membranes prevents them from being wetted with water. Moreover, the air trapped by the cones may well impede the lateral movement of water, a supposition based on our observations that the cones become waterlogged if the air is removed by suction prior to the ingress of water. It might be inferred that, in nature when only the occasional pore is flooded, the air would be forced out through the unflooded pores. It is notable, therefore, that the pores in the hen's egg appear to resist the outward movement of air (Haines and Moran, 1941). This view of the water repellancy of the cone layer is further supported by our observations (Table 2), that water-immiscible liquids of low surface tension rapidly flood the pores and the cone layer whereas ethanol and similar compounds were drawn into the shell membranes. This novel concept of the shell and its membranes is similar to the situation found in the plastron (Beament, 1961; Hinton, 1969), a device whereby insect embryos can develop without gills even though the egg is continuously or intermittently submerged in water.

Infection of eggs

When discussing their observations on the rotting of eggs, Gillespie and Scott (1950) surmised that " the overall probability of bacterial rotting . . . is doubtless

dependent upon many factors which affect the various stages of infection of the egg ". Shells are contaminated by contact with dirty surfaces (Rosser, 1942) and the contaminants are confined to the surface of the shell (Büchli, 1967). Fewer than 1% of nest clean eggs addle during extended storage (Brooks and Taylor, 1955) but there is often a high incidence of rotting in washed eggs (Gillespie *et al.*, 1950*a*; Winter *et al.*, 1955). It has been generally accepted that the shell provides a barrier to the invasion of the egg's contents by bacteria and that water is necessary for the translocation of organisms across the shell (Board, 1966). This view was supported by the present study. From a theoretical viewpoint, the infection of the shell membranes with rot-producing bacteria would appear to be dependent upon the number of rot-producing bacteria and their location on the shells.

Under natural conditions, a low incidence of pores liable to flooding would appear to call for a heavy and uniformly distributed contamination of the shell before there was a bacterium coincident with the orifice of a cuticle-less pore. Alternatively, an increase in the incidence of such pores would presumably reduce the need for either a high level or uniform distribution of the contaminants. In laboratory trials, it has been demonstrated repeatedly that the incidence or extent of contamination of the contents of eggs is directly related to the number of organisms used to challenge the shell (Lorenz *et al.*, 1952; Stokes *et al.*, 1956; Brant and Starr, 1962; Hartung and Stadelman, 1963).

In the present study the pronounced skewing of the distribution of carbon black penetration of the shell (Figs 1 and 2) indicates that with eggs coming from commercial flocks, there is an appreciable incidence of eggs having a large number of pores liable to bacterial penetration even if the level of contamination were low. A similar tailing was noted by Rauch (1952, 1954), who used a measure of the electrical resistance of the shell as an index of the diameter of the pores, there being a few eggs in each sample which had exceptionally low electrical resistance (large pores). In our limited observations, the incidence of eggs penetrated in many places with carbon black was higher in white than in brown eggs. It has been noted (Simons and Wiertz, 1963; Carter, 1969) that there is a marked variation in cuticle thickness. Could this account for the as yet unexplained observations (Gillespie and Scott, 1950; Trussell *et al.*, 1955) that brown eggs are more resistant to rotting than white eggs?

The high incidence of rotting in eggs washed in some of the original egg-washing machines (Gillespie *et al.*, 1950*b*) led to attempts to improve their design and operation (Brant *et al.*, 1966; Büchli, 1967). In such studies, the incidence of rotting is still used as an index of the efficiency of a machine in minimising microbial infection of the egg contents (Büchli, 1967). Our results suggest that carbon black could be used to establish whether or not a machine was promoting particle penetration of the shell.

It would be of interest to know if the cuticle-less (either partial or complete) egg is a characteristic trait of a hen or merely a chance happening in clutches laid by all hens. If the former, then in nature one might expect that the trait would be eliminated by the infection of the embryo. In the commercial hatchery, this selective pressure may be ameliorated by high standards of hygiene, fumigation, etc. Nevertheless, lack of cuticle may account in part for the otherwise inexplicable reason for certain eggs failing to hatch—Rauch (1952, 1954), for example, noted

that hatchability was low in eggs having pores of large diameter. In addition, the cuticle-less egg may account for certain practical problems. For example, do they account for the occasional failure in the sterilisation of eggs in gnotobiology (Harrison, 1969); are they a means whereby salmonellae are introduced in large numbers to egg products; could they be the vehicle which transmits pathogens from parent stock to specific pathogen free progeny (Harry and McLintock, 1972), and are they the " incubator clears " which have micrococci in their albumen (Seviour *et al.*, 1972)?

When considering addling of the egg, it would seem advisable to distinguish between penetration of the shell and colonisation of the contents. Thus, for example, the demonstrations (Lorenz *et al.*, 1952; Vadehra *et al.*, 1970*b*) that the incidence of rotting is higher in eggs which have been challenged at the broad pole may not be indicative of this part of the shell being more porous; it is more probably due to the earlier induction of rotting by the yolk uniting with the infected shell membranes at the air cell end (Board, 1964). Likewise the increased incidence of rotting of eggs having their cuticle removed (Vadehra *et al.*, 1970*a*) could be due not only to an increased incidence of infection of the shell membranes but to enhancement of microbial growth in the membranes by improved gaseous exchange through the shell and the increased levels of extraneous materials, such as iron or iron transport compounds, which would be deposited at the sites of infection of the shell membranes.

Elimination of pathogens from incubator eggs

Many workers have attempted to prevent transmission of pathogens from breeding flocks to progeny by injecting antibiotics into the eggs (Stuart and Keenum, 1970). In a review, Parry (1970) implied that the failure to ensure complete control limits the commercial application of the technique. A notable feature of these studies is the extensive variation in the uptake whether measured as weight of solution absorbed by each egg or concentration of antibiotic in each egg. Moreover, the use of coloured antibiotics has shown an incidence of shell penetration similar to that noted in Figs. 1 and 2 (Alls *et al.*, 1963, 1964). One of the principal advocates (Voeten, 1965) of antibiotic treatment of incubating eggs devised a system (direct pressure difference dipping), whereby the antibiotic was forced into the egg by the sudden release of a vacuum. In our experience (Figure 8), this does tend to decrease the extent of scatter in the amount of water absorbed by a sample of eggs but, judging from the distribution of pigment in treated eggs, the water is confined, at least initially, to the cone layer of the shell. The weight increase may not be a useful index of effective antibiotic uptake if the latter cannot diffuse into the white because of deposition on the inner surface of the shell. Nevertheless, the present study indicates that the overall efficiency of antibiotic absorption might be improved, as was suggested by Alls *et al.* (1964), if a small part of the shell was freed of cuticle and if the surface tension of the solvent could be reduced without harm to the embryo.

Conservation

The failure in breeding success of hawks, etc., has been attributed to DDT and related compounds interfering with shell formation (Ratcliffe, 1970). In addition

to thin shells it has been shown with quail that feeding DDT leads to a high incidence of large pores, apparently free of cuticle, and a low incidence of cones on the inner surface of the shell (McFarland *et al.*, 1971). This suggests that the water repellancy of the shell may be impaired and that it may be a poor barrier against microbial invasion and raises the question: are the eggs removed from hawks nests because they are broken or because they have rotted?

Formaldehyde mixed with the base of proprietary horticultural sprays have been used in attempts to control the spread of flocks of gulls. Although the embryo can be killed, subsequent rotting and explosion of the egg can lead to the parents rearing young from a second clutch of eggs. As flocks of gulls are normally a problem in inaccessible regions, the regular treatment of eggs is not possible. Thus there is a need for one application only during the breeding season. The present study has indicated that the efficiency of such control measures might be improved by the choice of a solvent which, through having a low surface tension, would rapidly carry through the shell substances toxic both to the embryo and micro-organisms.

Nomenclature

Following the demonstration (Baudrimont and Martin-St-Auge, 1847) that the shell of the hen's egg contains pores, many workers have attributed certain properties of the shell to " porosity ". The data given in Table 3 suggest that the term has not been interpreted exclusively in the sense defined by Marshall and Cruickshank (1938), and thus it has acquired a nebulous meaning. The following is offered as a basis for a revised terminology.

TABLE 3

Methods used to determine " porosity " of the shell of the hen's egg

Method	Investigator[1]
Loss in weight of stored eggs	Dunn (1923-24); Hays and Sumbardo (1926-27); Pringle and Barott (1937); Marshall and Cruickshank (1938); Tyler (1945)
Staining of shell	Nathusius (see Tyler, 1964); Rizzo (1899); Weston and Halnan (1927); Almquist and Holst (1931)
Release of gas by eggs in water under vacuum	Bryant and Sharp (1934)
Permeability of shells to:	
gases	Romanoff (1943); Haines and Moran (1941); Romijn (1950); Mueller (1958)
liquids	Alls *et al.* (1963, 1964)
particles	Paton and Ayres (1964)
Electrical conductivity of shell	Rauch (1952, 1954)
Retention of water by shells immersed in water	Romanoff (1943)

[1] Selected references only.

Porosity. This may be defined as " having pores " or " being permeable " (Fowler and Fowler, 1969). It would be convenient to use the former meaning and reserve it for holes which extend across the shell but not necessarily the cuticle (bloom) or cover (Tyler, 1969*b*). At this time, the method best suited for demonstrating such pores is uncertain. In our experience (Fig. 3), points of penetration by methylene blue from the inside-outside of the shell (hens) cleaned by boiling in 2·5% (w/v) NaOH for 15 min tended to give a count of the same order as that obtained with the same pieces of shell after they had been etched (Tyler, 1953) with nitric acid. We noted, however, that great care had to be taken in timing the acid etching otherwise the shell disintegrated or gave counts larger than those given by the staining method. Acid etching would not be appropriate for shells in which several pore canals terminate at a common orifice on the outer surface of the shell (Tyler, 1969*b*). Thus porosity might be reserved for the incidence of pore orifices at the outer surface of the column layer of the shell.

Transpiration. This may be defined as " to pass off vapour, moisture through pores of skin, etc." (Fowler and Fowler, 1969). When considering water loss by eggs, this term would appear to be more appropriate than the older term porosity, or a possible alternative, evaporation, which has a connotation of turning into vapour. As discussed by Tyler (1945) transpiration would have to be related to a defined area of shell and qualified by reference to factors such as temperature, water vapour pressure of the atmosphere, properties of shell and derivation of the egg in the sense of age, breed of hen, position in clutch, etc.

Gaseous exchange. There would appear to be a need for distinguishing between uptake of O_2 and loss of CO_2 and to consider separately the changes occurring in fertile and infertile eggs. With the former, for example, there might be a need to distinguish between the exchange occurring through the shell over the air space and that elsewhere (Romijn and Roos, 1938; Romijn, 1950; Visschedijk, 1968).

Absorption. This term could be used when considering the uptake of solvents or solutes by an egg. With the former, a weight change per unit area may be appropriate and, from the studies of Fromm (1959) and Fromm and Monroe (1960), it might be necessary to relate absorption to weight loss of the egg prior to treatment. Moreover, absorption would need to be qualified by reference to the surface tension of the liquid, period of exposure and hydrostatic pressure. With solutes, the partition of these within the egg would be important. Thus in our studies, it was noted that iron drawn through the pores of a contracting egg was retained by the ovotransferrin in the shell membranes.

Particle penetration. Although bacterial invasion of the shell has been studied extensively, the needs of asepsis have tended to make the investigators rely on indirect evidence of penetration (Williams and Whittemore, 1967). Although direct methods have been devised (Paton and Ayres, 1964; Board and Board, 1967), the requirement for incubation has curtailed their application. In the present study, the evidence showed that particles such as carbon black can be used to simulate bacterial invasion of the shell. Although more than 1000 eggs were examined, only some of the factors influencing particle penetration have been identified. Thus future work needs to be directed to investigation of factors which determine the extent of cuticle deposition and the changes (Simons and Wiertz, 1963) occurring in the cuticle during the storage of eggs, influence of shell thickness, etc.

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REFERENCES

ALLS, A. A., BENTON, N. J., KRAUSS, W. C. AND COVER, M. S. (1963). The mechanics of treating hatching eggs for disease prevention. *Avian Dis.*, **7**: 89-97.

ALLS, A. A., COVER, M. S., BENTON, W. J. AND KRAUSS, W. C. (1964). Treatment of hatching eggs for disease prevention—factors effecting permeability and a visual detection of drug absorption. *Avian Dis.*, **8**: 245-256.

ALMQUIST, H. J. AND HOLST, W. F. (1931). Variability of shell porosity in the hen's egg. *Hilgardia*, **6**: 61-72.

BAUDRIMONT, A. AND MARTIN-ST-AUGE, G. J. (1847). Recherches sur les phemomenes chimigues de evolution embryonaire des eiseau et des bactracenis. *Annls Chim. Phys.*, Sec. III. T. **21**: 195-295.

BEAMENT, J. W. L. (1961). The water relations of insect cuticle. *Biol. Rev.*, **36**: 281-320.

BEAN, K. C. AND MCLAURY, D. W. (1959). The bacterial contamination of hatching eggs and methods for its control. *Poult. Sci.*, **38**: 693-698.

BELLAIRS, R. AND BOYDE, A. (1969). Scanning electron microscopy of the shell membranes of the hen's egg. *Z. Zellforsch. mikrosk. Anat.*, **96**: 237-249.

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.*, **27**: 350-364.

BOARD, R. G. (1966). The course of microbial infection of the hen's egg. *J. appl. Bact.*, **29**: 319-341.

BOARD, R. G. (1968). Microbiology of the egg: a review. In: *Egg Quality: A study of the Hen's Egg*, pp. 133-162. Edit. CARTER, T. C. Edinburgh, Oliver and Boyd.

BOARD, R. G. (1969). The microbiology of the hen's egg. *Adv. appl. Microbiol.*, **11**: 245-281.

BOARD, P. A. AND BOARD, R. G. (1967). A method of studying bacterial penetration of the shell of the hen's egg. *Lab. Pract.*, **16**: 471, 472, 482.

BRANT, A. W. AND STARR, P. B. (1962). Some physical factors relating to egg spoilage. *Poult. Sci.*, **41**: 1468-1473.

BRANT, A. W., STARR, P. B. AND HAMANN, J. A. (1966). *Poultry Tribune*: P. 65.

BROWN, W. E., BAKER, R. G. AND NAYLOR, N. B. (1965). The role of the inner shell membrane in bacterial penetration of chicken eggs. *Poult. Sci.*, **44**: 1323-1327.

BRYANT, R. L. AND SHARP, P. F. (1934). Effect of washing on the keeping quality of hen's eggs. *J. agric. Res.*, **48**: 67-89.

BROOKS, J. AND TAYLOR, D. I. (1955). Eggs and egg products. *Rep. Fd Invest. Bd, 60*. London, Her Majesty's Stationery Office.

BÜCHLI, K. (1967). De invloed van het wassen op de microbiologische houdbaar-heid van het ei Instituut voor der Pluimveeteelt " Het Spelderholt "—Beekbergen Mededeling No. **145**.

CARTER, T. C. (1969). The hen's egg: relationship between the shell thickness and the amount of organic matter in the shell. *Br. Poult. Sci.*, **10**: 165-174.

CATTANEO, G. (1877). Sulla produzione di micofiti nell 'interno delle ova. *Atti Soc. ital. Sci. nat.*, **20**: 89-102.

COOKE, A. S. AND BALCH, D. A. (1970*a*). The distribution and carbohydrate composition of the organic matrix in hen egg shell. *Br. Poult. Sci.*, **11**: 353-365.

COOKE, A. S. AND BALCH, D. A. (1970*b*). Studies of membrane, mammillary cores and cuticle of the hen egg shell. *Br. Poult. Sci.*, **11**: 345-352.

DUNN, L. C. (1923-24). The variation of eggs in the rate at which they lose weight. *Poult. Sci.* **3**: 136-148.

FOWLER, F. G. AND FOWLER, H. W. (1969). *The Pocket Oxford Dictionary of Current English*, 5th ed., Oxford, Clarendon Press.

FROMM, D. (1959). A rapid method for the determination of egg shell permeability. *Poult. Sci.*, **38**: 171-173.

FROMM, D. AND MONROE, R. J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg shell permeability. *Fd Technol. Champaign.*, **14**: 401-403.

GILLESPIE, J. M. AND SCOTT, W. J. (1950). Studies in the preservation of shell eggs. IV. Experiments on the mode of infection by bacteria. *Aust. J. appl. Sci.*, **1**: 514-530.

GILLESPIE, J. M., SCOTT, W. J. AND VICKERY, J. R. (1950*a*). Studies in the preservation of shell eggs. II. The incidence of bacterial rotting in unwashed eggs and eggs washed by hand. *Aust. J. appl. Sci.*, **1**: 215-223.

GILLESPIE, J. M., SCOTT, W. J. AND VICKERY, J. R. (1950*b*). Studies in the preservation of shell eggs. III. The storage of machine washed eggs. *Aust. J. appl. Sci.*, **1**: 531-538.

HAINES, R. B. AND MORAN, T. (1941). Porosity of, and bacterial invasion through the shell of the hen's egg. *J. Hyg., Camb.*, **37**: 453-461.

HARRISON, G. F. (1969). Production of germ-free chicks: a comparison of the hatchability of eggs sterilized externally by different methods. *Lab. Anims*, **3**: 51-59.

HARRY, E. G. AND MCLINTOCK, M. (1972). The production of disease free embryos and chicks. In: *Safety in Microbiology*, pp. 121-132. Edit. SHAPTON, D. A. AND BOARD, R. G. London, Academic Press.

HARTUNG, T. E. AND STADELMAN, W. J. (1963). *Pseudomonas fluorescens* penetration of egg shell membranes as influenced by shell porosity, age of egg and degree of bacterial challenge. *Poult. Sci.*, **42**: 147-150.

HAYS, F. A. AND SUMBARDO, A. M. (1926-27). Physical characters of eggs in relation to hatchability. *Poult. Sci.*, **6**: 196-200.

HINTON, H. E. (1969). Respiratory system of insects. *A. Rev. Ent.*, **14**: 343-368.

KRAFT, A. A., MCNALLY, E. H. AND BRANT, A. W. (1958). Shell quality and bacterial infection of shell eggs. *Poult. Sci.*, **37**: 638-644.

LACK, D. (1968). *Ecological Adaptations for Breeding in Birds*. London, Methuen.

LIFSHITZ, A., BAKER, R. G. AND NAYLOR, N. B. (1964). The relative importance of chicken egg exterior structure in resisting bacterial penetration. *J. Fd Sci.*, **29**: 94-99.

LORAH, M. E., FUNK, E. M. AND FORWARD, J. (1954). Reducing spoilage in shell eggs by the use of fungicides. *Missouri agric. Exp. Stn. Res. Bull.*, **573**.

LORENZ, F. W., STARR, P. B., STARR, M. P. AND OGASAWARA, F. X. (1952). The development of *Pseudomonas* spoilage in shell eggs. I. Penetration through the shell. *Fd Res.*, **17**: 351-360.

MARSHALL, W. AND CRUICKSHANK, D. B. (1938). The function of the cuticle in relation to the porosity of eggs. *J. agric. Sci., Camb.*, **28**: 24-42.

MCFARLAND, L. Z., GARRETT, R. L. AND NOWELL, J. A. (1971). Normal egg shells and the egg shells caused by organochlorine insecticides viewed by the scanning electron microscope. *Proc. 4th A. Scanning Electron Microsc. Symp., Chicago*, 377-384.

MUELLER, W. J. (1958). Shell porosity of chicken eggs. 1. CO_2 loss and CO_2 content of infertile eggs during storage. *Poult. Sci.*, **37**: 437-444.

NEWALL, V. (1971). *An Egg at Easter, a Folklore Story*. Routledge and Kegan Paul: London.

PARRY, R. T. (1970). Dipping hatching eggs in antibiotic solutions to reduce the spread of mycoplasma infections. *Poult. Rev.*, **80**: 90-95.

PATON, A. M. AND AYRES, J. C. (1964). Use of a fluorescent brightners for tracing passage of *Salmonella* in eggs. *Nature, Lond.*, **204**: 803-804.

PRINGLE, E. M. AND BAROTT, H. G. (1937). Loss of weight of hens' eggs during incubation under different conditions of humidity and temperature. *Poult. Sci.*, **16**: 49-52.

RATCLIFFE, D. A. (1970). Changes attributable to pesticides in egg breakage frequency and egg shell thickness in some British birds. *J. appl. Ecol.*, **7**: 67-115.

RAUCH, W. (1952). The influence of egg shell porosity on the number of chickens hatched from incubating eggs. *Poult. Sci.*, **31**: 589-594.

RAUCH, W. (1954). Biophysikalische Messungen an Bruteiern. *Arch. Geflügelk.*, **18**: 297-304.

RIZZO, A. (1899). Sul numero e sulla distributione jei pori nel guscio dell'ovo di gallina. *Richerchie del Anat. Las. Roma.*, **7**: 171-199.

ROMANKEWITSCH, N. A. (1934). Das Kutikulum des Huhnereies. *Z. Zellforsch. mikrosk. Anat.*, **21**: 110-118.

ROMANOFF, A. L. (1931). Why some eggs do not hatch. *Cornell Univ. Bull.* No. 205.
ROMANOFF, A. L. (1943). Study of various factors effecting permeability of birds' eggshells. *Fd Res.*, **8**: 212-223.
ROMANOFF, A. L. (1967). *Biochemistry of the Avian Embryo.* New York, Wiley.
ROMANOFF, A. L. AND ROMANOFF, A. J. (1949). *The Avian Egg.* New York, Wiley.
ROMIJN, C. (1950). Foetal respiration in the hen. Gas diffusion through the egg shell. *Poult. Sci.*, **29**: 42-51.
ROMIJN, C. AND ROOS, J. (1938). The air-space of the hen's egg and its changes during the period of incubation. *J. Physiol. Lond.*, **94**: 365-379.
ROSSER, F. T. (1942). Preservation of eggs. II. Surface contamination on egg shell in relation to spoilage. *Can. J. Res.*, **20D**: 291-296.
ROSSER, F. T., WHITE, W. H., WOODCOCK, A. H. AND FLETCHER, D. A. (1942). Preservation of eggs. I. Treatment for maintaining quality in shell eggs at ordinary temperatures. *Can. J. Res.*, **20D**: 57-70.
SEVIOUR, E. M. AND BOARD, R. G. (1972). The behaviour of mixed bacterial infections in the shell membranes of the hen's egg. *Br. Poult. Sci.*, **13**: 33-43.
SEVIOUR, E. M., SYKES, F. R. AND BOARD, R. G. (1972). A microbiological survey of the incubated eggs of chickens and waterfowl. *Br. Poult. Sci.*, **13**: 549-556.
SHARP, P. F. (1932). Detection of washed, abrased and oiled eggs. *Industrial and Engineering Chemistry*, **24**: 941-950.
SHARP, P. F. AND STEWART, G. F. (1936). Effect of relative humidity on the growth of mold on eggs in storage. *Cornell Univ. Agric. Exp. Sta. Memoir* **191**.
SIMONS, P. C. M. AND WIERTZ, G. (1966). The ultra-structure of the surface of the cuticle of the hen's egg in relation to egg cleaning. *Poult. Sci.*, **45**: 1153-1162.
SMITH, A. J. M. (1934). The relations between yolk and white in the hen's egg. VI. The equilibrium between ice and egg-yolk, and the freezing-point difference between yolk and white. *J. exp. Biol.*, **11**: 228-242.
STOKES, J. L., OSBORNE, W. W. AND BAYNE, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Fd Res.*, **21**: 510-518.
STUART, E. E. AND KEENUM, R. D. (1970). Preincubation treatment of chicks hatching eggs infected with *Salmonella pullorum*. *Avian Dis.*, **14**: 87-95.
TRUSSELL, P. C., FULTON, C. O. AND CAMERON, C. J. (1955). Bacterial spoilage of shell eggs. II. Incidence of spoilage in eggs from 94 farms. *Fd Technol., Champaign*, **19**: 130-134.
TYLER, C. (1945). The porosity of egg shells, and the influence of different levels of dietary calcium upon porosity. *J. agric. Sci.*, **35**: 168-176.
TYLER, C. (1953). Studies on egg shells. II. A method of marking and counting pores. *J. Sc. Fd Agric.*, **4**: 266-273.
TYLER, C. (1955). Studies on egg shells. VI. The distribution of pores in egg shells. *J. Sci. Fd Agric.*, **3**: 170-176.
TYLER, C. (1956). Studies on egg shells. VII. Some aspects of structure as shown by plastic models. *J. Sci. Fd Agric.*, **7**: 483-493.
TYLER, C. (1964). A translated and edited version of the work of Wilhelm von Nathusius on Avian Eggshells. The University, Reading.
TYLER, C. (1969*a*). The distribution of pores in the egg shells of the domestic fowl: a further study. *Br. Poult. Sci.*, **10**: 375-380.
TYLER, C. (1969*b*). Avian eggs: their structure and characteristics. *Int. Rev. gen. exp. Zool.*, **4**: 82-127.
VADEHRA, D. V., BAKER, R. G. AND NAYLOR, N. B. (1970*a*). Role of cuticle in spoilage of chicken eggs. *J. Fd Sci.*, 35: 5-6.
VADEHRA, D. V., BAKER, R. G. AND NAYLOR, N. B. (1970*b*). Infection routes of bacteria in chicken eggs. *J. Fd Sci.*, 35: 61-62.
VISSCHEDIJK, A. H. J. (1968). The air space and embryonic respiration. 3. The balance between oxygen and carbon dioxide in the air space of the incubating chicken egg and its role in stimulating pipping. *Br. Poult. Sci.*, **9**: 197-210.
VOETEN, A. C. (1965). Control of chronic respiratory disease in chickens by egg dipping in a solution of tylosin tartrate. *Ph.D. thesis, University of Utrecht.*

WALDEN, C. C., ALLEN, I. V. F. AND TRUSSELL, P. C. (1956). The role of the egg shell and shell membranes in restraining the entry of microorganisms. *Poult. Sci.*, **35**: 1170-1196.

WESTON, W. A. R. D. AND HALNAN, E. T. (1927). " Black spot " of eggs. *Poult. Sci.*, **6**: 251-258.

WILLIAMS, J. E., DILLARD, L. H. AND HALL, G. O. (1968). The penetration patterns of *Salmonella typhimurium* through the outer structure of chicken eggs. *Avian Dis.*, **12**: 445-466.

WILLIAMS, J. E. AND WHITTEMORE, A. D. (1967). A method for studying microbial penetration through the outer structure of avian eggs. *Avian Dis.*, **11**: 467-490.

WINTER, A. R., BURKART, B., CLEMENTS, P. AND MACDONALD, L. (1955). Cleaning eggs with detergent and detergent sanitizers. *Ohio Agric. Exp. Stn Res. Bull.*, **762**.

WOLKEN, J. J. (1951). Structure of the hen's egg membranes. *Anat. Rev.*, **111**: 79-89.

ZAGAEVSKY, J. S. AND LUTIKOVA, P. O. (1944). Sanitary measures in the egg breaking plant. *U.S. Egg Poult. Mag.*, **50**: 17-20, 43-46, 75-77, 89-90, 121-124.

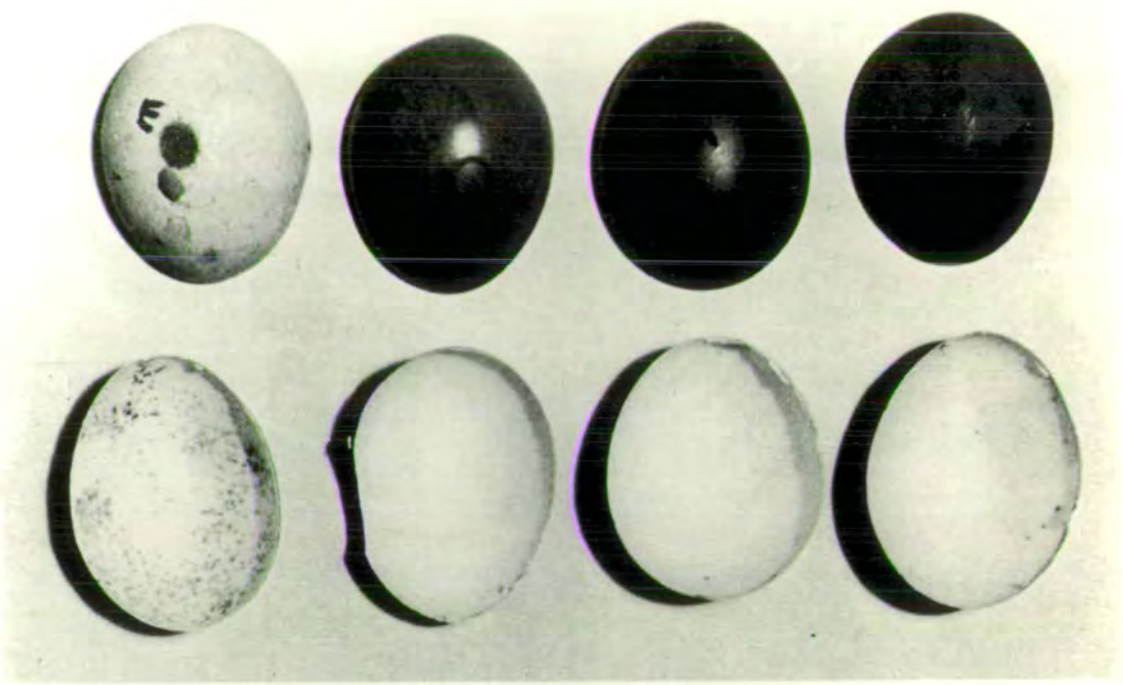

PLATE-FIG. 1.—Eggs which had been immersed in a chilled solution of Edicol pea green containing carbon black. Top row: left-hand egg, shell freed of cuticle with EDTA did not stain and the shell membranes (left-hand egg, bottom row) contained many spots of carbon black. The other eggs had well formed cuticle and (bottom row) few spots of carbon black in their shell membranes.

PLATE-FIG. 2.—Eggs which had supported the growth of a streptomyces did not stain evenly with Edicol pea green.

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RESEARCH NOTE

WATER UPTAKE BY EGGS OF MALLARDS AND GUINEA FOWL

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SYNOPSIS

The cuticle is the principal barrier to water uptake by the eggs of mallard, guinea fowl and the domestic fowl. The fat present in the cuticle of mallard eggs makes a large contribution to repelling water dropped on to the shell but only a small contribution to resisting water movement into eggs exposed to hydrostatic pressure. Although the thickness of the shells of the eggs of the birds mentioned above varied extensively, there was no obvious correlation of the length of the pore canal with the amount of water taken up by an egg.

INTRODUCTION

The cuticle's role in waterproofing the shell of the hen's egg has been discussed in a previous report (Board and Halls, 1973). The present communication presents evidence of the cuticle being involved in the waterproofing of the shell of the eggs of mallard (*Anas platyrhynchos* L) and guinea fowl (*Numidia meleagrus*).

MATERIALS AND METHODS

The mallard eggs were collected from nests at the Wildfowl Trust Slimbridge and the guinea fowl eggs were produced by a small, mated flock on free range. The hen eggs were obtained from an unmated flock (housed in batteries) of a commercial flock of brown-egg layers. They were 3 to 4 d old at the time of examination.

The cuticle on half the shell was removed by partially submerging eggs for 15 or 30 min in a 10% (w/v) solution of ethylenediamine-tetraacetic acid (EDTA) adjusted to pH 7·5. Control eggs were held for an equal period in distilled water. The wetted surface of the shell was held under running tap water and rubbed vigorously with paper tissues. Eggs were weighed to within 0·1 mg, placed in a domestic pressure cooker and covered with 1% (w/v) solution of Edicol Supra pea green H (ICI Hexagon House, Blackley, Manchester) containing 0·1% (v/v) of carbon black vs (ICI). A positive pressure of 34·5 mN/mm^2 15 min was imposed by connecting the pressure cooker to the control valve on a cylinder of H_2 (95 parts) CO_2 (5 parts). The eggs were dried with tissues and weighed. The shell was bisected longitudinally and the number of coloured patches on the shell membrane enumerated.

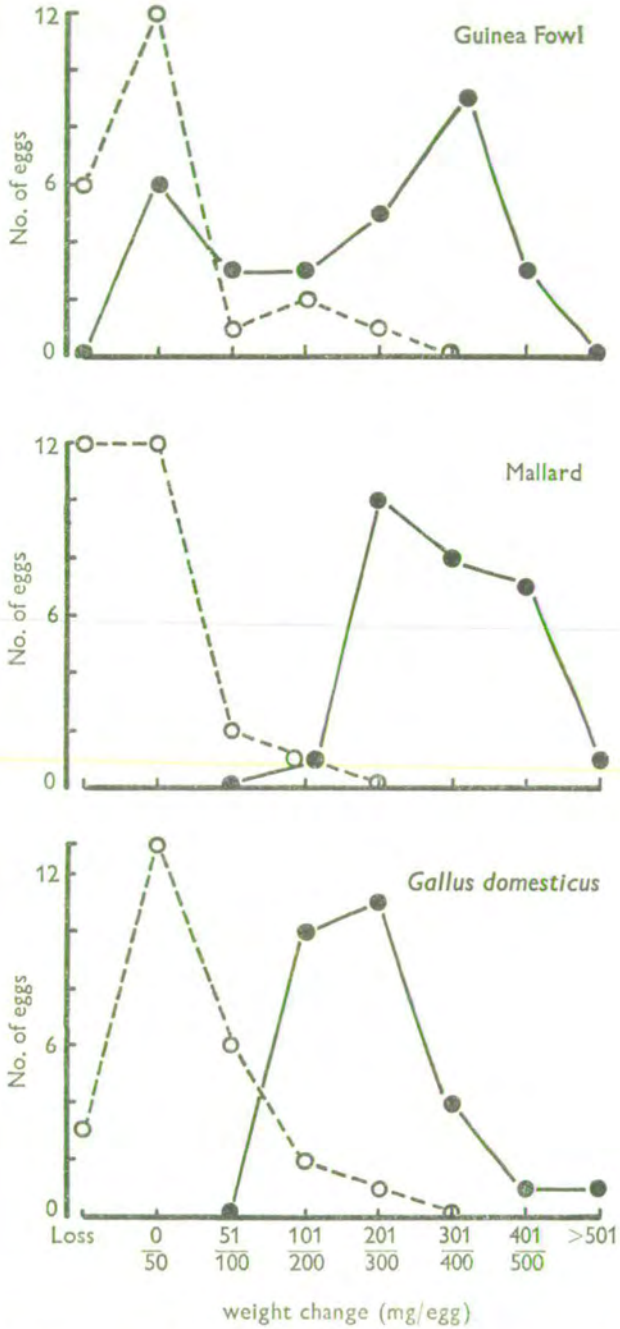

FIG. 1.—Water uptake by eggs. Open circles, control eggs, and closed circles, eggs from which the cuticle had been removed from half the shell

RESULTS AND DISCUSSION

The results are summarised in Fig. 1. The profiles of the graphs given by EDTA-treated and control eggs of domestic hen are similar to those discussed by Board and Halls (1973). These results provide further evidence of the role of the cuticle in impeding water uptake by hen eggs exposed to hydrostatic pressure. The results obtained with untreated guinea fowl eggs were similar to those for control eggs of domestic hens. EDTA treatment of the shell of guinea fowl eggs led to a marked increase in water uptake by the majority of eggs. The cuticles on these eggs required 30 min exposure to EDTA and vigorous rubbing with paper tissues before

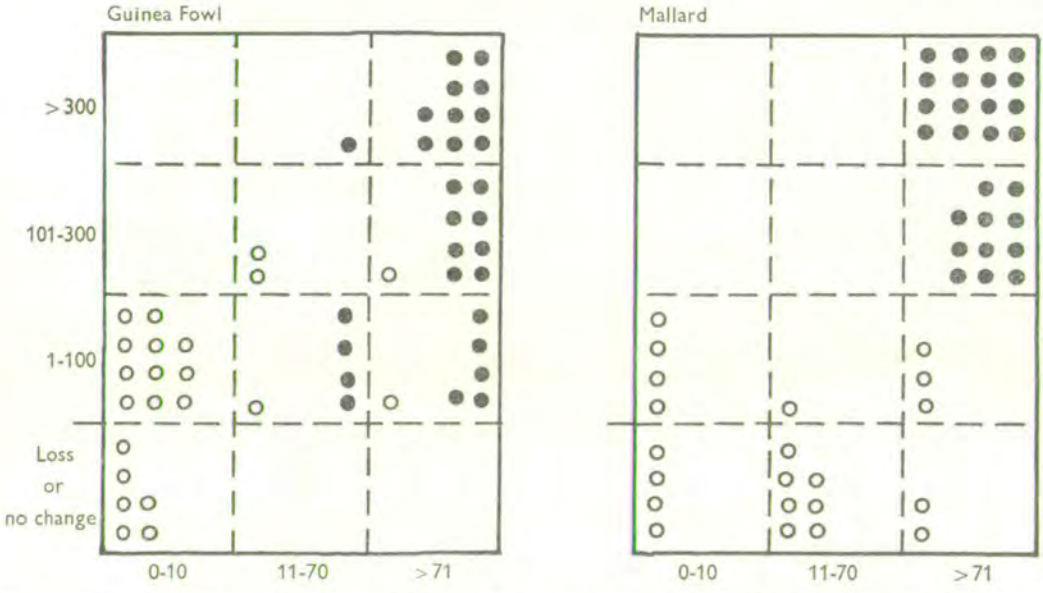

FIG. 2.—Water uptake and carbon black penetration of the shells of eggs. Open circles, control eggs, and closed circles, eggs from which the cuticle had been removed from half the shell.

they could be removed. Even then, the surface of the treated shell contained many brown spots which were assumed to be pore canals from which cuticle had not been removed. It is suggested that cuticular plugging of the pore canals was responsible for some of the eggs (the secondary peak in Fig. 1) failing to take up water. Further evidence of there being material within the pores which impeded the movement of water through the shell came from counts of the number of pigmented patches on the shell membranes of EDTA-treated guinea fowl eggs (Fig. 2). EDTA-treatment did not result in all the shells being penetrated in more than 70 places. The pattern given by EDTA-treated shells of domestic hens was essentially the same as that shown for mallard eggs in Fig. 2. The shells of the guinea fowl were thicker (mean of 30 random measurements, 1·49 mm) than those of the domestic hens' eggs (mean of 30 measurements, 0·767 mm). This suggests that the length of the pore canal plays, if at all, a secondary role to the cuticle in impeding the passage of water across the shell.

The cuticle on mallard eggs was easily removed with EDTA and rubbing and, judging from the number of pores penetrated (Fig. 2), the cuticle was removed from the pore orifice. The uptake of water by the EDTA-treated eggs (Fig. 1) was of the same order as with the cuticle-less eggs of domestic hens. Of the eggs used in this study the mallard had the thinnest shells (mean of 30 measurements, 0·656 mm). This provides further evidence that the length of pore canal plays a negligible role in impeding water movement into eggs. The results obtained (Fig. 1) with control mallard eggs gave a pattern different from those of domestic hens or guinea fowl—the number of mallard eggs losing weight equalled those whose weight did not change during immersion in water with a hydrostatic pressure of 34·5 mN/mm^2 15 min. The majority of mallard eggs which lost weight (Fig. 2) had from 11 to 71 stained patches on the shell membrane. Thus it would seem that, in addition to physical blocking of the pore orifices, another property (oiliness?) of the cuticle contributed to the water repellancy of the eggs.

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REFERENCE

BOARD, R. G. AND HALLS, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poult. Sci.*, **14**: 69-97.

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CUTICLE, SHELL POROSITY AND WATER UPTAKE BY HENS' EGGSHELLS

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Running headline : CUTICLE AND HENS' EGGSHELLS

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1. There was no correlation between water uptake by a warm egg (37°C) immersed in iced water and the shells porosity as measured by water vapour conductance.

2. Eggs lacking cuticle on the shell took up more water than those having cuticle. There was, however, no correlation between water up-take and the water vapour conductance of cuticle-less eggshells.

3. In general there was no correlation between the amount of water taken up by an egg and the weight of cuticle on its shell. With some eggs there was an association between water up-take and the extent of fissuring of the cuticle.

4. It was postulated that the pore canals impede water flow such that osmotic forces acting across the shell membranes do not influence water up-take by eggs.

INTRODUCTION

It is generally accepted that a very small percentage, probably less than 1%, of hens' eggs contain micro-organisms at oviposition (Brooks and Taylor, 1955). Thus the breaching of the physical defence offered by the egg's integument (the calcitic shell with its outer covering of cuticle, the inner and outer shell membranes and the limiting membrane) can be considered to be the first step in the process leading to the rotting of table or incubated eggs (Board, 1966), in the genesis of omphalitis (Harry, 1957) and, perhaps ultimately even the colonization of the alimentary canal, especially the caeca, of newly hatched chicks with salmonellae (Snoeyenbos et al., 1978). Although there are suggestions in the literature that the integument is readily breached immediately following oviposition (Zagaevsky and Lutikova, 1944), the majority of studies have been concerned with eggs which were challenged with bacteria following removal from nest boxes.

Water, either as vapour or liquid, appears to be essential for microbial penetration of the integument. Thus a high (95% R.H.) water content in the atmosphere surrounding an egg is a prerequisite for mould or bacterial growth on the cuticle and subsequent microbial penetration of the pores in the calcitic shell (Board et al., 1979). The studies of Haines and Moran (1941) identified some of the factors which contribute to microbial penetration of the shells of eggs immersed in water. They noted a low rate of penetration when water and eggs were of the same temperature but a high rate when the eggs were warmer than the water. The latter observation led them to conclude that a negative pressure was created in the cooling egg because the volume of the contents contracted more than that of the shell, the difference being satisfied when contaminated water was sucked through the pores in the shell. Indeed their observations

have had a major impact on practices in the poultry industry. Thus in the washing of table eggs, emphasis is given to the need to avoid the generation of a negative pressure - the temperature of the wash water must be greater than that of the eggs (Moats, 1978). An opposing strategy is adopted to free incubating eggs of mycoplasma, warm eggs being immersed in chilled solutions of antibiotics (Alls *et al.*, 1963, 1964).

Although factors contributing to microbial penetration of the eggs integument have been known for more than 40 years, little headway has been made in attempts to identify the contribution of the various components, especially their physical attributes, of the integument to the egg's defence. Romanoff (1931) appears to have concluded intuitively that the cuticle played a major role; supporting observations were published by Vadehra *et al.* (1970) and Board and Halls (1973). Other workers (e.g. Williams *et al.*, 1968) have concluded that the resistance of the inner shell membrane to microbial penetration is greater than that of the outer one. Such conclusions must be cautious because the experiments did not distinguish between the resistance of the inner shell membrane *per se* and the limiting membrane (Tranter *et al.*, in press). As was noted by Board & Halls (1973) interpretation of many of the studies of bacterial penetration of egg shells is difficult through the common use of the term, porosity, which has been rendered nebulous because of many different definitions. The classical studies of the diffusion of water vapour across avian egg-shells by Rahn and his collaborators (Ar *et al.*, 1974) have provided the poultry industry with a definition of this term and a simple means of determining this attribute of a shell.

This communication presents observations on factors affecting water uptake by hens' eggs of known porosity *sensu strictu*. The study was based on the hypothesis that water uptake, as measured by a gain in weight, provides an index of the effectiveness of an egg's integument in preventing penetration by microorganis-

MATERIALS AND METHODS

Eggs

Eggs were obtained from an unmated commercial flock (Ross ranger) housed in batteries. The eggs were used on the day of collection.

Water vapour conductance

Water loss from the egg was expressed according to the equation (Ar et al., 1974) :

$$G_{H_2O} = \frac{M_{H_2O}}{\Delta P_{H_2O}}$$

where G_{H_2O} = Water vapour conductance of the eggshell expressed as mg of water lost per mmHg difference in water vapour pressure across the shell per day.

M_{H_2O} = Water loss from the egg in mg per day.

ΔP_{H_2O} = Difference in water vapour pressure across the eggshell in mmHg.

G_{H_2O} is a measure of the number and size of the pores in the eggshell and it is a constant property once shell formation has been completed.

G_{H_2O} was determined by packing preweighed eggs in desiccant (Drierite 10/20 mesh; Koch Light Laboratories Ltd., Colnbrook, Bucks., England) contained in a vented desiccator maintained at 37°C. The eggs were repacked in fresh desiccant daily and reweighed on day 3. As the water vapour pressure around the eggs was 0mmHg and the egg contents were assumed to be at the saturated vapour pressure (for the relevant temperature), ΔP_{H_2O} may be determined and subsequently G_{H_2O}.

Water uptake

A modified technique based on principles described by Haines and Moran (1941) and Board and Board (1967) was used. Preweighed eggs (at 37°C) were immersed for 15 minutes in chilled (1°C) water or a 10g/1ℓ solution of Edicol supra pea green H (I.C.I., Hexagon House, Blackley, Manchester, England). The eggs were removed after 15 minutes and weighed immediately after the shells had been dried by wiping.

Cuticle staining and removal

The cuticle was stained with Edicol supra pea green H as described by Board and Halls (1973). The eggs were immersed for 15 minutes in a 0.34M alkaline (pH 7.5) solution of ethylenediaminetetraacetic acid (EDTA). The cuticle was removed with a fine jet of water.

Electron microscopy

Samples of shell were placed in liquid nitrogen and then freeze dried (Edwards, Crawley, Sussex, England). They were mounted on aluminium planchettes and sputter coated *in vacuo* with a thin layer of gold/palladium alloy. The samples were examined with a Jeol 35C (Jeol (UK) Ltd., Colindale, London, England) scanning electron microscope.

Some samples of shell fixed in 0.5M gluteraldehyde in 0.2M cacodylate buffer (pH 7.0) for 60 minutes at room temperature were dehydrated in a series (50-100%) of acetone solutions. The samples were dried using a critical point dryer (Polaron Equipment Ltd., Watford, London, England) and mounted on aluminium planchettes. They were coated *in vacuo* with a gold/palladium alloy.

Cuticle crack length

Samples of shell were freeze dried and micrographs of the cuticle taken using a Jeol 35C S.E.M. at a magnification of X130. A transparent film with a ruled grid (9 x 6 squares) was laid over the micrograph and the cuticle cracks traced for the required (statistically decided) square, on to tracing paper. Crack length was measured with a map measure (Polco Products Ltd., Brentford, Middx., England).

Pore counts and distribution

Acid etching, as used by Tyler (1969), was used to determine pore numbers and their distribution was assessed by the method of Tyler and Fowler (1978). This involves the measurement of nearest neighbours (r_A) in a known area of shell (A). From the mean ($\bar{r}_A$), a value Q' was determined :

$$Q' = \frac{A}{\pi\ (\bar{r}_A{}^2)}$$

When Q' is plotted against N (number of cones or pores), a random distribution will give a line bisecting the origins of the axes with a slope of 1.00. Points above this line is taken as evidence of aggregation whilst those below of uniformity.

Cone counts and distribution

Cone counts were made by the method of Tyler and Fowler (1978) except that scanning electron micrographs were used instead of drawings made with a projection microscope.

Shell-less eggs

Shell-less eggs from commercial flocks were immersed in water. At regular intervals they were taken out of the water, dried with a towel and weighed.

Shell Thickness

An anvil-jawed micrometer (hemispherical jaws) was used to measure shell thickness of shells from which the membranes had been removed, the mean of 3 readings obtained from separate shoulder pieces being taken.

RESULTS

Although measurement of water vapour conductance by the method of Ar et al. (1974) can be used to determine the porosity of an eggshell, the actual location of the pores in the integument of a hen's egg needs to be stressed. The outer orifices of the pores are covered and in many instances roughly plugged with the cuticle - a fissured layer formed from spheres of glycoproteins (Wedral et al., 1974). The inner orifices are located in clefts between the cones, the tips of which fuse with the outer of the two shell membranes. These are elastic in nature and behave as semipermeable membranes (Figure 1).

When untreated eggs at 37°C were immersed in ice cold water for 15 minutes, the amount of water taken up ranged from 3 - 415 mg/egg (number of eggs 90). There was no correlation, however, between the amount of water taken up by an egg and the porosity of the shell (Figure 2). Likewise there was no correlation when the number of pores per shell - 8-20 x 10^3 - was regressed against water uptake ($r = 0.03$; $P>0.05$: number of eggs, 70). Additional evidence that pores per se had little influence on water

uptake by warm eggs in chilled water came from an analysis of the distances between pores and pore numbers and the distances between cones and cone numbers (Table 1). This information led us to conclude that eggshells having relatively few (large) but widely distributed pores were not especially vulnerable to flooding with water and vice versa. In practice, of course, this conclusion obtains only when all the pores, upwards of 10,000 per egg, are considered. Subsequent studies with chilled solutions of Edicol pea green showed that very large patches of shell membrane were stained in some eggs and it was inferred that these patches occurred at the internal orifice of a pore having a large canal which had been formed as a consequence of a localized malformation in the cone layer. Such pore canals have been referred to as "patent pores" (see Board, 1980). Likewise large patches of stained membrane were commonly found underlying a part of the shell having a calcareous lump projecting from its surface. These observations led us to conclude that such lumps, which appear on occasions to be persistent traits of the eggs of particular hens cause malformation of the pores in the adjoining shell.

The lack of evidence associating porosity with water uptake led to an examination of the cuticle. Ice cold solutions of Edicol pea green were used in these studies. It is evident from Figure 2 that warm eggs which had been freed of cuticle by EDTA took up larger amounts of water than did the untreated controls. As the G_{H_2O} of the former did not differ significantly from the latter ($P>0.05$), the chelating agent had obviously not modified appreciably the pore canals. The use of the stain led to the identification of certain factors that negate the water resistance conferred on shells by the cuticle. Thus one or two malformed pores in a shell clothed with cuticle caused appreciable water uptake by an egg. Some of the eggs in which there was an appreciable weight increase (up to 415 mg) following immersion did not have cuticle on the entire shell or one or other of its

poles - indeed naturally cuticle-less eggs behaved in the same way as those from which cuticle had been removed with EDTA. A poor cuticle was a characteristic feature of the eggs of some hens (e.g. No.8) at the end of their laying cycle (Fig.3). Others had damaged cuticles, commonly a scrape which we assumed had been caused by an egg with the moist cuticle of oviposition being damaged as it rolled down the inclined wire netting floor of the batteries. The incidence of such eggs was about 40% of the eggs included in this study. The use of the stain showed also that large amounts of water were taken up by eggs having hair-line cracks in their shells. No correlation was evident when the amount of water taken up by an egg was regressed against the amount of cuticle per cm^2 shell ($r = 0.50$; $P>0.05$: number of eggs 48). The latter property was determined by removing the cuticle with EDTA, harvesting it on a glass microfibre filter and, after adequate washing, drying to constant weight.

The extent of fissuring of the cuticle was considered to be a potential cause of the failure to demonstrate an association between water uptake by an egg and cuticle quality as determined gravimetrically. This surmise was tested thus. The water uptake by 50 eggs was determined and five eggs selected as representatives of those having high, intermediate or low water uptake. The length of the fissures in the cuticle of these eggs was measured (see Materials and Methods) and regressed against water uptake by the respective eggs. A significant correlation was noted ($r = 9.0$; $P<0.05$). No such correlation was evident, however, when the fissure length in the cuticles of 24 eggs were regressed against the amount of water each had taken up. The practical implications of these observations will be considered in the Discussion. Through determining the contact angle (ϕ), the dimensions of the fissures as well as the microstructure of their radial faces will obviously influence the effectiveness of the cuticle as a barrier to water. It is noteworthy, therefore, that the effectiveness of the cuticle was

diminished when a surfactant was added to water (Figure 4a). This figure shows also that water uptake was increased by degassing the water; this indicates presumably that under practical conditions some of the gas comes out of solution at the water:cuticle interface thereby satisfying in part the pressure differential generated by a warm egg contracting in cold water.

The evidence discussed above identifies cuticle as the major impediment to water penetration into the hen's egg. It is not, however, the sole contributor to waterproofing. If it were, then a clear-cut relationship between water uptake and G_{H_2O} of shells freed of cuticle by EDTA would be expected (Figure 2). Indeed these results indicate that some feature of the pore canals plays a role, albeit minor, in impeding water movement. Although this attribute has not been identified unequivocally, some of our observations suggest that resistance to water flow - a drag factor - may be involved. The semi-permeable and elastic properties of the shell membranes of naturally shell-less eggs are evident in the results presented in Figure 1. Thus there would appear to be the potential for the column of water in a flooded pore canal to be pulled inwards by osmotic forces acting across the shell membranes. Indeed the large areas of stained membranes, together with appreciable water uptake by eggs whose shells contained malformed pores or hair-line cracks, is taken to be evidence of osmotic forces acting across the shell membrane. Likewise the tendency of a surfactant (Figure 4a) to enhance water uptake by eggs may well be evidence of a diminution of the drag factor postulated above.

DISCUSSION

Discussions of the biological consequence of G_{H_2O} is concerned with two conflicting requirements of the embryo, a porosity that allows sufficient gaseous diffusion to support embryogenesis but that does not cause excessive water loss and hence dehydration of the egg. Studies of the hatching eggs of commercial flocks of turkeys and hens have shown (Tullett, 1981, Tullett and Burton, 1982) that the majority of eggshells have a porosity within a range that accommodates this conflict. The embryos in very porous shells can be protected from excessive water loss through humidity control of the incubator; those in shells at the other extreme will be asphyxiated. Although porosity has a central role in the incubating egg, this study is the first to demonstrate that it has a negligible influence on the amount of water taken up when a warm egg contracts in iced water. Indeed the results presented in this paper support the conclusion (Board and Halls, 1973) that the cuticle confers water resistance to the hen's egg. Moreover as the cuticle does not influence G_{H_2O} appreciably, a breeding programme concerned with the latter need not involve the former.

The biological function(s) of the cuticle on the hen's egg have attracted little attention. In the context of avian eggs in general Lack (1968) surmised that material on the outer surface of the calcitic shell was an adaptation that fitted eggs laid in wet places to an inimical environment - he did not specify the dangers to which the embryos would be exposed should the shell lack such adaptations. In a discussion of Lack's (1968) hypothesis, Board (1982) suggested that, from the viewpoint of gaseous diffusion, the integument on avian eggs can be considered to be a series of diffusion pathways (or resistances) and that modifications of the outer surface of the shell are adaptations that ensure optimal gaseous diffusion in nests containing debris that could possibly occlude the pore canals. Such a role for the cuticle was evident in a study of guinea fowl eggs incubated in the

wild (Board and Perrott, 1982). We contend that the present communication is the first to demonstrate conclusively that the cuticle on the hen's eggshell does not influence G_{H_2O} but that it does protect the underlying pore canals from flooding and thus, by implication, bacterial contamination. These functions of the cuticle have important practical implications also. The water resistance conferred on the shell by the cuticle poses a problem to those who wish to rid incubating eggs of mycoplasma by flooding at least some of the pores with antibiotics (Alls et al., 1964). Alternatively attempts to prevent egg transmission of Salmonella spp. from breeding stock to broiler, for example, may be negated by imperfectly formed or damaged cuticle allowing the organisms to penetrate the shell, especially of eggs laid in dirty nest boxes or on the floor.

There is little evidence about the synthesis of cuticle in domestic hens and only one study (Ball et al., 1975) which suggests that cuticle "quality" may be a heritable factor. Only one method - contraction of a warm egg in iced water - was used in attempts to overcome the cuticles' resistance to water because it is the principal cause commercially of bacterial infection of eggs, or at least those challenged subsequent to collection from the nest (Tranter and Board, 1982). In practice the present study identified the role of cuticle through experiments in which water uptake by normal eggs was compared with those which were cuticle-less at oviposition or had been rendered cuticle-less by EDTA. The significant differences between such eggs suggest that a simple staining reaction could be used in a breeding programme designed so that the incidence of naturally cuticle-less eggs was minimised. The results obtained from a survey of eggs laid by individually caged birds suggest that it would be a useful technique to follow gross changes in cuticle deposition during a laying cycle. Our observations of extensive water uptake by some eggs having large fissures in the cuticle suggests that a method of assessing cuticle quality other than

its staining potential would be needed to select for those that were particularly effective in endowing hens' eggshells with water resistance. In addition it needs to be recognised that malformation of the shell can undermine the function of the cuticle, "patent pores" and calcareous lumps penetrating the surface of the shell were two malformations identified in the present study. The latter appeared from a limited survey to be a trait of the particular hen and, if heritable, capable of being selected out from a flock. The cause of "patent pores" is unlikely to be known in detail until all the factors contributing to core formation, cone initiation and pore growth have been established and probably even then the complexities of shell initiation may well be such that an occasional pore of above average size will be formed in an otherwise normal egg.

ACKNOWLEDGEMENTS

One of us (NHCS) wish to thank the Ministry of Agriculture, Fisheries and Food for a studentship. We wish to thank Dr. V.D. Scott for allowing extensive use of the electron optic facilities and the Science and Engineering Research Council, U.K., for funding the purchase of many of these facilities.

REFERENCES

ALLS, A.A., BENTON, N.J., KRAUS, W.C. and COVER, M.S. (1963). The mechanics of treating hatching eggs for disease prevention. Avian Diseases, 7: 89-97

ALLS, A.A., COVER, M.S., BENTON, W.J. and KRAUS, W.C. (1964). Treatment of hatching eggs for disease prevention - factors affecting permeability and a visual detection of drug absorption. Avian Diseases, 8: 245-256.

AR, A., PAGANELLI, C.V., REEVES, R.B., GREENE, D.G. and RAHN, H. (1974). The avian egg: water vapour conductance, shell thickness and functional pore area. Condor, 76: 153-158.

BALL, R.F., LOGAN, V. and HILL, J.F. (1975). Factors affecting the cuticle of the egg as measured by intensity of staining. Poultry Science, 54: 1479-1484.

BOARD, P.A. and BOARD, R.G. (1967). A method of studying bacterial penetration of the shell of the hen's egg. Laboratory Practice, 16(29): 471-472, 482.

BOARD, R.G. (1966). Review article; the course of microbial infection of the hen's egg. Journal of Applied Bacteriology, 29: 319-341.

BOARD, R.G. (1980). The avian eggshell - a resistance network. Journal of Applied Bacteriology, 48: 303-313.

BOARD, R.G. (1982). Properties of avian eggshells and their adaptive value. Biological Reviews, 57: 1-28.

BOARD, R.G. and HALLS, N.A. (1973). The cuticle: A barrier to liquid and particle penetration of the shell of the hen's egg. British Poultry Science, 16: 69-91.

BOARD, R.G., LOSEBY, S. and MILES, V.R. (1979). A note on the microbial growth on hen eggshells. British Poultry Science, 20: 413-420.

BOARD, R.G. and PERROTT, H.R. (1982). The fine structure of the outer surface of the incubated eggshells of the Helmeted guinea fowl (Numida meleagris). Journal of Zoology (London), 196: 445-451.

BROOKS, J. and TAYLOR, D.I. (1955). Eggs and egg products. Report of the Food Investigation Board No.60, London, H.M.S.O.

HAINES, R.B. and MORAN, T. (1941). Porosity of, and bacterial invasion through the shell of hen's egg. Journal of Hygiene, 37: 453-461.

HARRY, E.G. (1957). The effect on embryonic and chick mortality of yolk contamination with bacteria from the hen. Veterinary Record, 69: 1433-1441.

LACK, D. (1968). Ecological adaptations for breeding in birds. London, Methuen.

MOATS, W.A. (1978). Egg washing - A review. Journal of Food Protection, 41: 919-925.

ROMANOFF, A.L. (1931). Why some eggs do not hatch. Cornell University Bulletin No.205.

SNOEYENBOS, G.H., WEINACK, O.M. and SMYSER, C.F. (1978). Protecting chicks and poults from salmonellae by oral administration of "normal" gut microflora. Avian Diseases, 22: 273-287.

TRANTER, H.T. and BOARD, R.G. (1982). The antimicrobial defence of avian eggs: Biological perspective and chemical basis. Journal of Applied Bacteriology, 4: 295-338.

TULLETT, S.G. (1981). Theoretical and practical aspects of eggshell porosity. Turkeys, 29(4): 24-28.

TULLETT, S.G. and BURTON, F.G. (1982). Factors affecting the weight and water status of the chick at hatch. British Poultry Science, 23: 361-269.

TYLER, C. (1969). The distribution of pores in the eggshells of the domestic fowl: a further study. British Poultry Science, 10: 375-380.

TYLER, C. and FOWLER, S. (1978). The distribution of organic cores, cones, cone junctions and pores in the eggshells of wild birds. Journal of Zoology (London), 186: 1-14.

VADEHRA, D.V., BAKER, R.C. and NAYLOR, H.B. (1970). Role of cuticle in spoilage of chicken eggs. Journal of Food Science, 35: 5-6.

WEDRAL, E.M., VADEHRA, D.V. and BAKER, R.C. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of Gallus gallus. Comparative Biochemistry and Physiology, 47B: 631-640.

WILLIAMS, J.E., DILLARD, L.H. and HALL, G.O. (1968). The penetration patterns of Salmonella typhimurium through the outer structure of chicken eggs. Avian Diseases, 12: 445-466.

ZAGAEVSKY, J.S. and LUTIKOVA, P.O. (1944). Sanitary measures in the egg breaking plant. U.S. Egg Poultry Magazine, 50: 17-123.

TABLE 1. The relationship between pore numbers, cone numbers and their distribution in hens' eggshells*

Relationship of distribution factor† versus	Correlation coefficient	Student t test (P)	Number of eggs examined
Number of pores	0.90	<0.001	46
	0.96	<0.001	9
Number of cones	0.95	<0.001	11
	0.93	<0.001	29

* The analytical methods of Tyler and Fowler (1978) were used - see Materials & Methods.

† Distribution factor Q' was determined from :

$$\frac{A}{\pi\ (\bar{r}_A{}^2)}$$

Where A = surface area; $\bar{r}_A{}^2$ = mean of nearest neighbour measurements of cones or pores. A correlation of 0.90 accounts for 81% of the variation ($0.90^2 \times 100$).

LEGEND

Figure 1

Water uptake by a shell-less egg. The egg was immersed in water and the weight increase measured (A). The egg was then removed and allowed to stand overnight before being immersed a second time (B).

Figure 2

Removal of cuticle (□) does not change the G_{H_2O} values compared with untreated (O) eggs although water uptake is increased.

Figure 3

Diagrammatic representation of the cuticle quality as determined by staining with Edicol Supra Pea Green H.

Figure 4a

Lowering the water's surface tension with a surfactant, Triton X-100 (●) leads to an increase in water uptake compared with the controls (O). Degassing (Fig.4b) the water (●) has a similar effect, controls (O). (— — control mean; --- degassed mean).

Fig.1

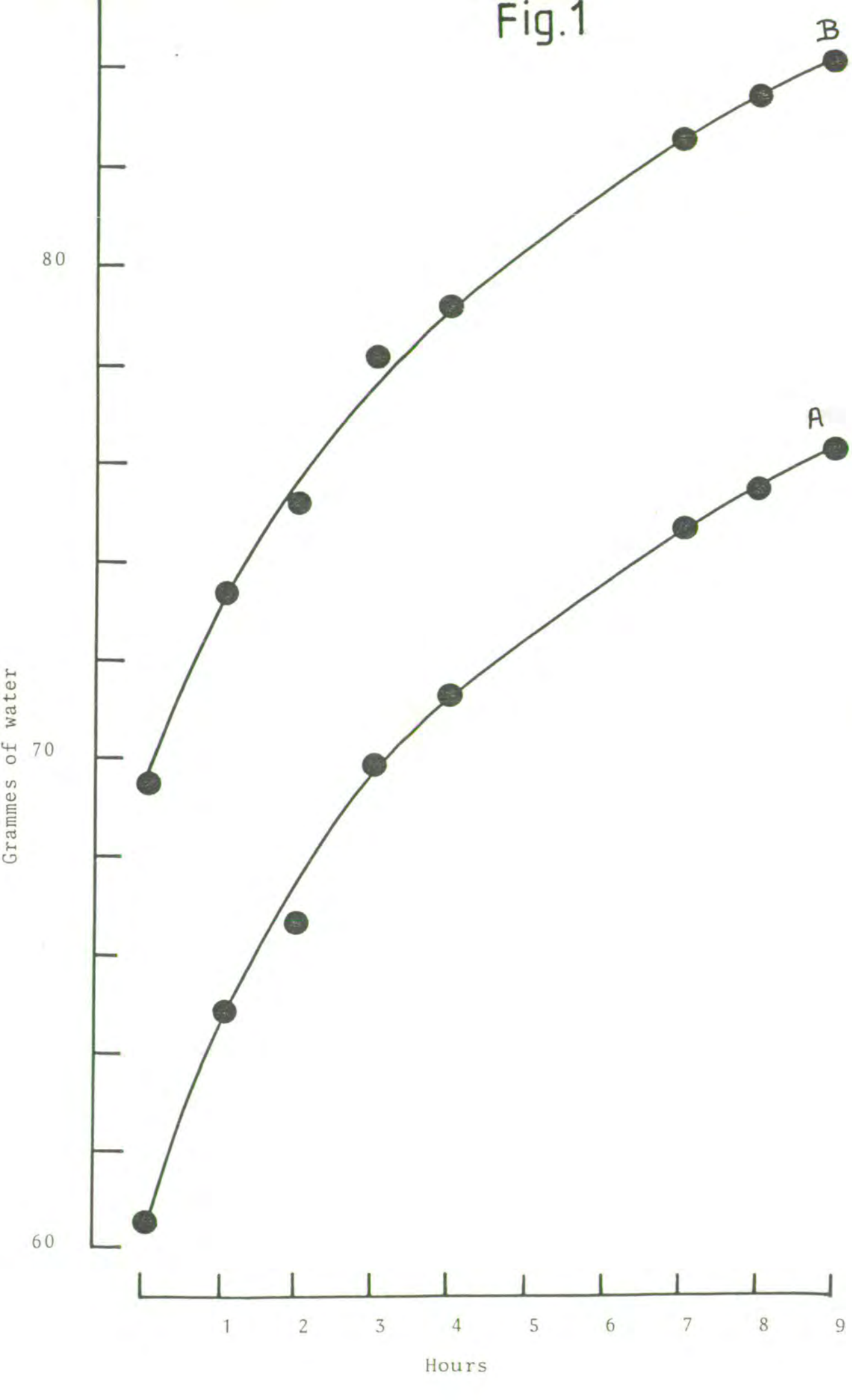
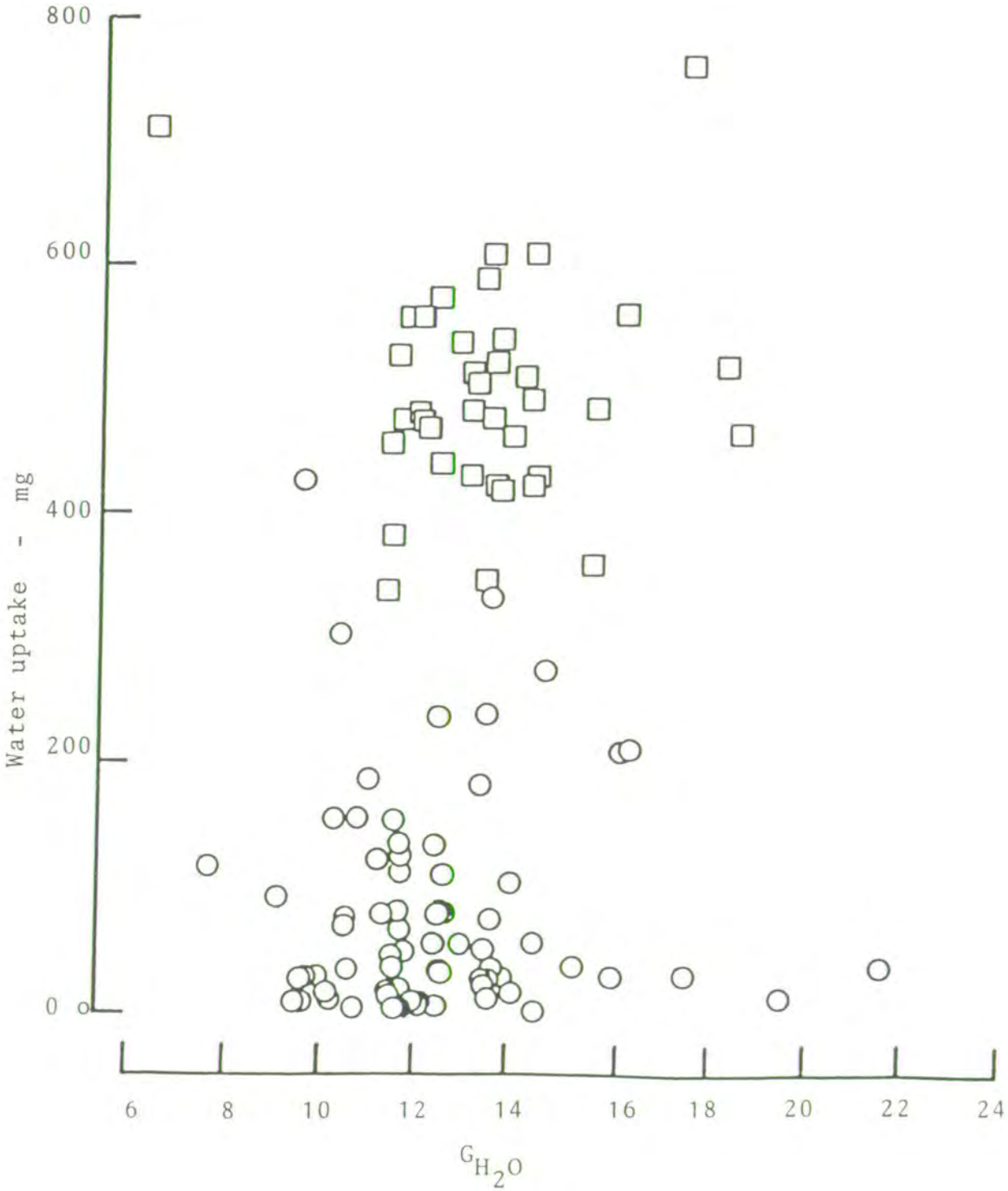


Fig.2



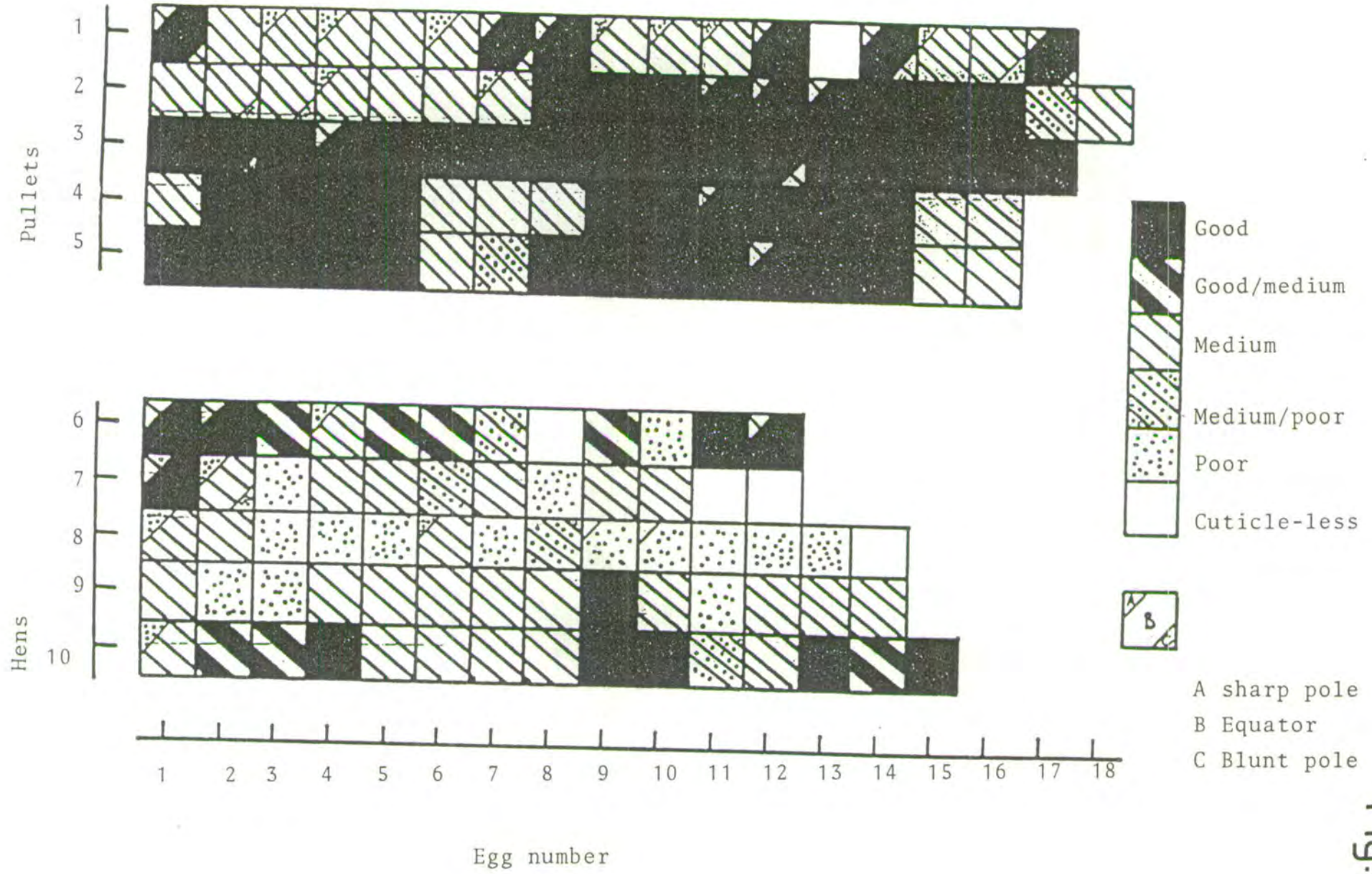
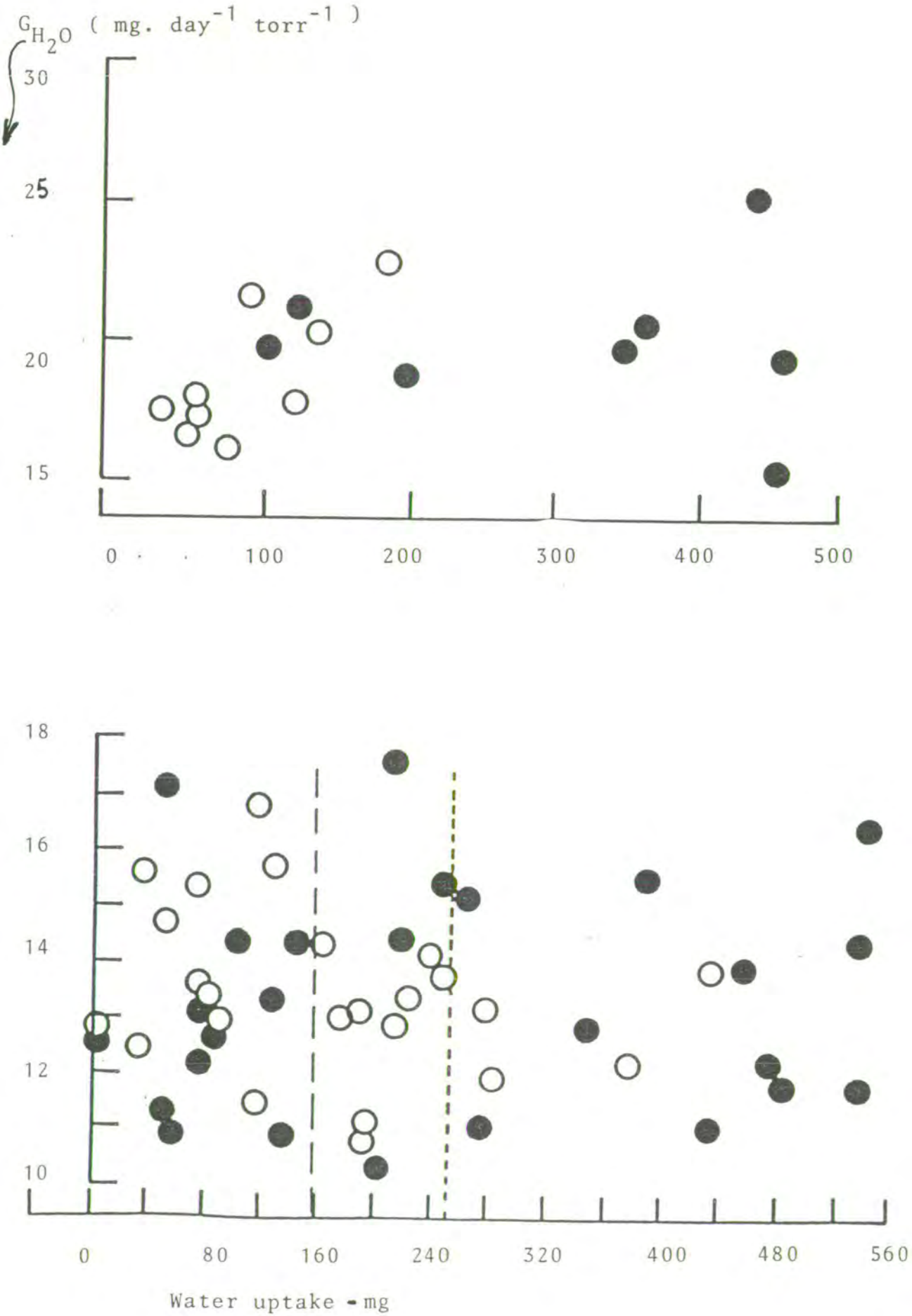

Pullets
1
2
3
4
5
Hens
6
7
8
9
10
Good
Good/medium
Medium
Medium/poor
Poor
Cuticle-less
A
B
A sharp pole
B Equator
C Blunt pole
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Egg number

Fig. 3

Fig. 4


G_{H_2O} (mg. day^{-1} torr^{-1})
30
25
20
15
0
100
200
300
400
500
18
16
14
12
10
0
80
160
240
320
400
480
560
Water uptake - mg

LABP 16-29 ARTICLE

A Method of Studying Bacterial Penetration of the Shell of the Hen's Egg

by Patricia A. Board and R. G. Board,
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Abstract

A technique is described in which the shells of intact eggs are challenged with known numbers of micro-organisms under controlled conditions.

Une méthode d'étude de la pénétration bactérienne de la coquille des oeufs de poule

On décrit une méthode dans laquelle les coquilles d'oeufs intactes sont soumises à l'attaque d'un nombre connu de micro-organismes dans des conditions contrôlées.

Eine Methode zum Studium bakterieller Durchdringung der Hühnereierschale

Eine Methode wird beschrieben, bei der die Schalen intakter Eier unter gesteuerten Verhältnissen dem Angriff einer bekannten Zahl von Mikroorganismen ausgesetzt werden.

Introduction

BACTERIAL penetration of the shell is considered to be the first major step in the process leading to microbial deterioration of eggs intended for human consumption (Gillespie and Scott 1950). Likewise, gross penetration of the shell of hatching eggs is a prerequisite in outbreaks of salmonellosis (Wilson 1945) and mushy chick disease (Harry 1957) and it may contribute to microbial contamination of products such as frozen whole egg. It is surprising, therefore, that the actual process of penetration has not been studied in detail. As a result the advisability of washing dirty eggs remains a controversial issue, the assessment of germicides intended for the sanitation of egg shells is based on indirect and possibly inappropriate methods, and the design of cleaning equipment is approached empirically. The present report is concerned with a technique whereby the shells of intact eggs are challenged with known numbers of micro-organisms under controlled conditions. Organisms that penetrate the shell are encouraged to grow by bathing the shell membranes in nutrients supplemented with tetrazolium. This material is reduced by the micro-organisms and the insoluble product, formazan, is deposited in the shell membranes. Thus the actual sites

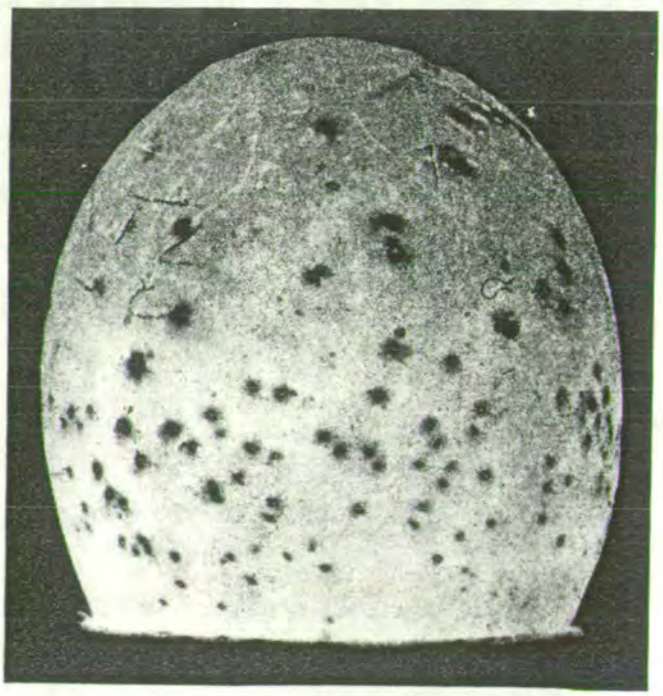

Figure 1. An agar-filled egg viewed with transmitted light.

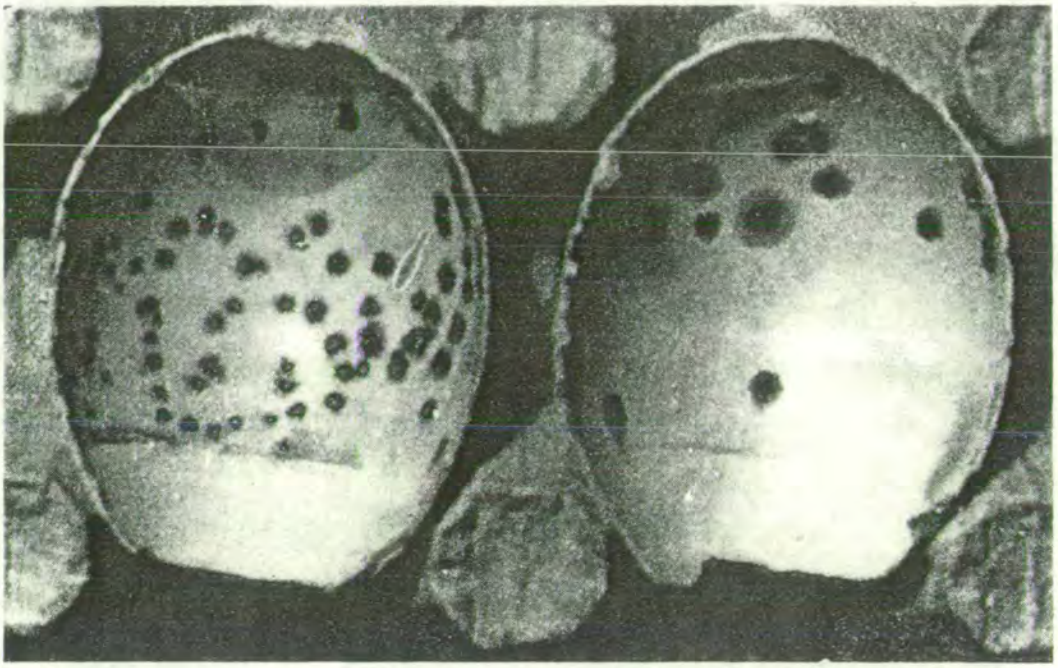

Figure 2. Inner surface of shell showing discrete spots of formazan at the sites of bacterial penetration.

of penetration are indelibly marked and detailed examination of the chemical and physical characteristics of the shells can proceed as time permits.

Method

Eggs obtained during one day from hens of known age and breed were candled and those having cracked or checked shells discarded. The sound eggs were placed on Keyes trays and held overnight at 27 C.

Gram-negative bacteria obtained from this Department's collection of stock cultures were used in this work. They were stored on slopes of nutrient agar (Oxoid) at 4 C and transferred at six-monthly intervals. The organism was grown on slopes of nutrient agar at 27 C and cells were harvested in 2 ml of sterile, quarter-strength Ringer's solution and the suspension shaken vigorously to break up clumps of cells. The suspension was then added to 500 ml of distilled water in a 2 litre stainless steel beaker: this resulted in a final population of 1.0 10×10^5 micro-organisms/ml.

Ten or twenty eggs were randomly taken from those held overnight at 27 C. They were gently lowered into the bacterial suspension and six cubes of ice added. To avoid contamination the operatives wore "dispos-a-glove" (Ethicon Ltd.). It is known (Haines and Moran 1940) that bacterial penetration of the shell is assisted by the contraction of a warm egg in cold water. After a definite period of immersion (normally 10 or 15 min) the eggs were removed and the shells dried with a hair dryer. When completely dried, the pointed ends of the shells were removed by means of an electrically driven carborundum-disc. The contents were allowed to drain from the shell and the inner surface of the shell membrane was flushed with sterile water. The empty shells were then filled with a medium of the following composition: glycerol, 0.5% (w/v); yeast extract (Oxoid), 0.05% (w/v); agar (Davis, New Zealand), 2.0% (w/v); tetrazolium (B.D.H.), 0.01% (w/v); tap water; pH 7.2. When the agar had set the hole in the shell was closed with sterile paraffin wax and the eggs, with their broad ends uppermost, were placed on Keyes trays and incubated at 27 C.

Results

The method of sealing the eggs allowed the growth of micro-organisms to be followed by the normal method of candling (Figure 1). The sealed end of the egg was put into the aperture of the candling lamp: light was transmitted through the agar and shell and formazan derived from microbial reduction of the tetrazolium was seen as discrete spots. When satisfactory growth of the organisms had occurred (usually by the second day of incubation) the shells were cut longitudinally. Figure 2 illustrates the appearance of the inner surface of the shell membrane. It was found that the formazan was deposited in both the inner and outer shell membranes and on the *inner* surface of the shell thus ensuring a permanent record of the actual sites of penetration.

Microbial growth within the agar was never observed, from which it was concluded that the spots of formazan arose from the reduction of tetrazolium by micro-organisms that had penetrated the shell and become enmeshed in the shell membranes during the time that the whole egg was immersed in the bacterial suspension. This supposition received support from experience with control eggs. These were randomly selected from those held overnight at 27 C and immersed in a bacterial suspension of the same temperature (*i.e.*, 27 C). They were dried with a hair dryer, emptied and the contents replaced with agar described previously. In no instance was growth of organisms found within the agar and growth in the shell membranes occurred only rarely. Thus it would appear that a generalized contamination of the inside of the egg does not occur during the preparative stages of this technique.

Because of the observation (Board 1964; 1966) that the shell membranes *in situ* do not allow optimum growth of the commonly occurring contaminants of rotten eggs, the method of Miles and Misra (1938) was used to compare microbial growth on shell membranes supported by the agar noted above and that occurring on nutrient agar (Oxoid). This investigation indicated that

(*Continued on page* 482)

Continued from page 472

single organisms could initiate growth on the shell membranes and their rate of growth, as indicated by development of the colony, equalled that of organisms on nutrient agar.

The technique described in this report has been used successfully with the following organisms: *Serratia marcescens*, a fluorescent pseudomonad and a coliform. Thus it would appear to be well suited to an intensive study of shell penetration by the commonly occurring contaminants of rotten eggs and has the advantage over the method of Paton and Ayres (1964) in that the sites of penetration can be observed without recourse to special illumination.

ACKNOWLEDGEMENT.—*We are indebted to the Director British Egg Marketing Board for financial assistance.*

REFERENCES

Board, R. G. (1964) *J. appl. Bact.* **27** 350.
Board, R. G. (1966) *J. appl. Bact.* **29** 319.
Gillespie, J. M. and Scott, W. J. (1950) *Aust. J. appl. Sci.* **1** 514.
Haines, R. B. and Moran, T. (1940) *J. Hyg. Camb.* **40** 453.
Harry, E. G. (1957) *Vet. Rec.* **69** 1433.
Miles, A. A. and Misra, S. S. (1938) *J. Hyg., Camb.* **38** 732.
Paton, A. M. and Ayres, J. C. (1964) *Nature, Lond.* **204** 803.
Wilson, J. E. (1945) *Vet. Rec.* **57** 411.

SECTION 4

THE NEST ENVIRONMENT

The notion that the avian eggshell functions as a mediating boundary between the developing embryo and the nest/bulk environment directs attention to the actual environment obtaining in the nest and its possible interaction with the general environment of the nesting site. Although the breeding biology of birds has been studied in great detail, the emphasis has been placed mainly on bird behaviour. Indeed it was the lack of detailed information about the environment of the nest cup that led to the work reported in two of the off-prints presented in this section. The first of these deals with the development of a sophisticated radiotelemetry system for monitoring the environment of the nest cup. The other presents the results obtained in the first extensive studies with the equipment.

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Editor: H. P. KIMMICH, Nijmegen
Publishers: S. KARGER, Basel
REPRINT (Printed in Switzerland)

Biotelemetry *4:* 169–180 (1977)

A Pulse-Position-Modulated Multichannel Radio Telemetry System for the Study of the Avian Nest Microclimate

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The Wildfowl Trust, Martin Mere, Lancs.

Key Words. Nest microenvironment · Multichannel biotelemetry · Multiparameter biotelemetry · Pulse position modulation · Humidity · Transponder · Data-logging

Abstract. A multichannel biotelemetry system using pulse position modulation has been developed to monitor continuously the environment in birds' nests. Probes to measure temperature at six points, relative humidity, light and egg attitude, together with a transmitter and power supply are contained in a fibreglass shell, having the shape, size and weight of the egg of the species being studied. The sensing egg is placed in the nest at the completion of a clutch and not removed until the young have hatched. A low-power transponder is used to relay the signals up to 2 miles to a data-logging system in the laboratory.

Introduction

There have been several studies of the temperature regime of the avian nest by various remote-recording techniques [KOSSACK, 1947; SCHWARTZ, 1977; VARNEY, 1974]. Although it has been possible to 'interpret' more than just temperatures from these results, the understanding of the nest microclimate has been limited by the lack of simultaneous information on such parameters as the position of the sensors, movement of the eggs, attentiveness of the parent and the relative humidity of the nest atmosphere.

The availability of low-cost CMOS integrated circuits has permitted the construction of multichannel transmitters which are capable of functioning for long periods on small batteries. The system described here makes maximum use of these devices. A nine-channel transmitter that

will operate continuously for periods in excess of 3 months, although weighing less than 10 g when complete with batteries, is described. The low drain of current from the batteries is due to the inherent low power consumption of the CMOS devices and the choice of pulse position modulation (PPM), the relative merits of which have been discussed by KIMMICH [1975]. This form of modulation does not transmit data in a truly simultaneous mode; when the response time constants of the sensors are considered, however, this is not likely to pose a problem. With a mean frame duration of 150 msec, the information from each sensor is updated seven times a second.

The demultiplexed PPM signal is readily convertible to a digital form using crystal-controlled pulse-timers. This technique ensures high, as well as long-term, calibration stability of the decoders (within 10 p/10^6) since it is dependent solely on the frequency stability of the reference crystal. The calibration stability characteristics and resolution of most sensors do not approach the capability of this form of decoding.

A pulse transponder, transmitting on ultra-high frequency from a fixed Yagi antenna, has enabled data to be collected from nests in such remote locations as small islands. Even if the transmitted pulses vary in duration, this technique cannot corrupt the data as only the leading edges of the pulses are used to clock the demultiplexer.

Theory of Operation and Decoding

Theory of Operation

A single frame of information consisting of ten pulses (100-μsec duration) of RF as transmitted by this system is shown in figure 1. The time interval between the negative edge of one pulse and the positive edge of the following pulse carries the information on that channel. Since changes in the signal strength can lead to distortion of the decoded pulse width by the receiver, it is better in practice to use only the positive edge of each pulse to index the channels.

Two monostables and a decimal output decade counter (fig. 2) form the basic encoder to generate nine data channels and one synchronization channel. The two monostables (fig. 2, A, B) are connected to form a variable mark space ratio multivibrator. The period of A is fixed at 100 μsec while that of B depends on the state of the sensor switched into its timing circuit by the decade counter C. The counter is advanced by one for each

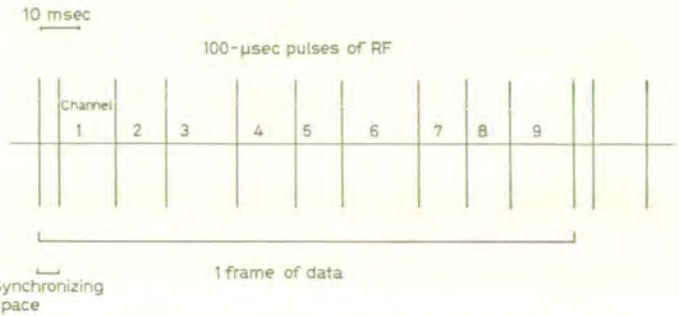


Fig. 1. A single frame of data, consisting of ten 100-μsec pulses of RF.

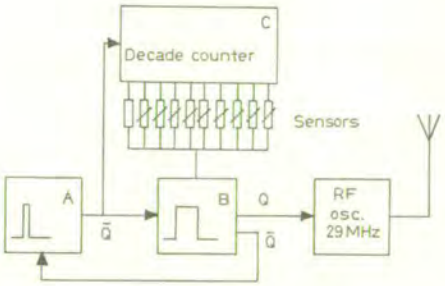


Fig. 2. The basic encoder; to generate nine data and one synchronization channel.

cycle of the multivibrator and resets automatically to zero after the ninth pulse, hence producing a continuous train of ten independently variable pulses. To achieve synchronization, one output of the counter feeds a fixed resistor selected to give a pulse shorter than the others. The shorter pulse is recognised by the receiver. The output of the encoder switches the transmitter on for the 100-μsec spacers between the variable pulses. The resulting output from the transmitter is a series of 100-μsec bursts of RF with one fixed synchronizing space per frame of ten pulses (fig. 1).

In this basic form, the encoder will only interface with sensors having an output in the form of a change in DC resistance, e.g. thermistors, photo-resistors, switched resistors, etc. To use the transmitter with a sulphonated polystyrene relative humidity probe, which changes in impedance with RH and is damaged by a DC current, the circuit shown in figure 3 has been developed. A low-frequency oscillator (50 Hz) feeds the sensor

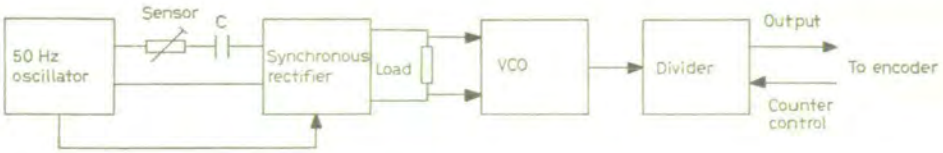
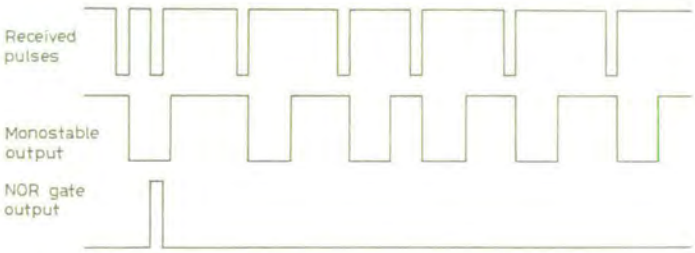


Fig. 3. Schema of the relative humidity sensor interface circuit. C = DC-blocking capacitor.



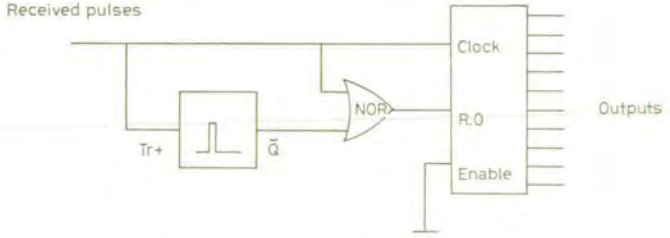


Fig. 4. The demultiplexer, and associated pulse diagram.

and a load resistor via an isolating capacitor. A synchronous rectifier, placed before the load and locked to the oscillator frequency, allows a DC voltage, varying with the impedance of the sensor and hence RH, to appear across the load. This voltage controls a VCO which, in turn, feeds a divider, controlled by the basic encoder, such that it starts counting from zero when this channel is selected. When a count of 2^7 is reached, the output of the divider goes high, allowing current to flow into the timing pin of monostable B. This completes that cycle of the multivibrator and ad-

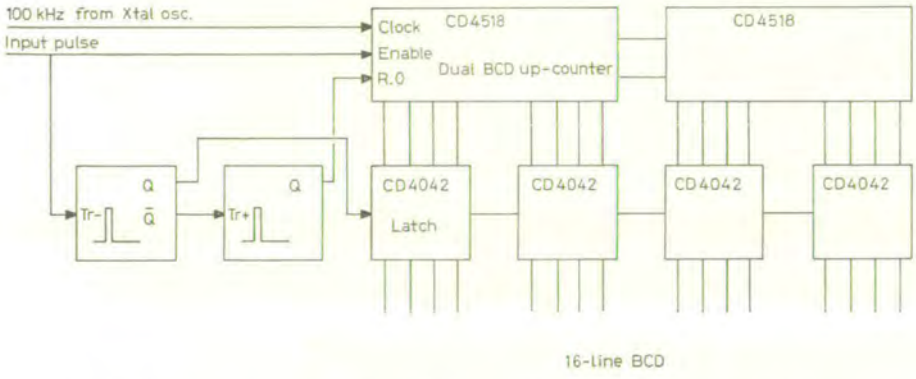


Fig. 5. Timer-counter module: ten of these units are used to convert the received pulse lengths to 16-line BCD.

vances the counter onto the next channel. Since the divide ratio is fixed, the period to fill the divider is dependent on the frequency of the VCO and hence RH.

Decoding

The received pulse train is demultiplexed using a counter similar to that in the transmitter and a monostable to detect the synchronization pause (fig. 4). After being converted to logic-compatible levels and inverted, the received pulses feed the counter and their trailing edges trigger the monostable. This is set to give a pulse of 5-μsec duration, which is shorter than the data pauses but longer than the synchronization pause. The monostable $\overline{Q}$ output and the incoming pulse train feed a dual input NOR gate. It will be seen from the pulse diagrams (fig. 4) that this produces a reset-zero pulse for the counter whenever a synchronization pause is transmitted. With the decoding counter synchronized to the incoming pulse train, the serial information is transferred to the ten parallel outputs of the counter. It appears on each of these as pulses at a rate dependent on the state of the other channels. The pulses on the ten parallel output lines are converted to 16-line BCD using ten identical timer-counter modules (fig. 5). These use a common 100-kHz clock frequency, derived from a 1-MHz crystal oscillator which allows pulses up to 99.99 msec to be measured. The reset-zero and latch pulses are derived from the trailing edges of the input pulses, using a pair of cascaded monostables.

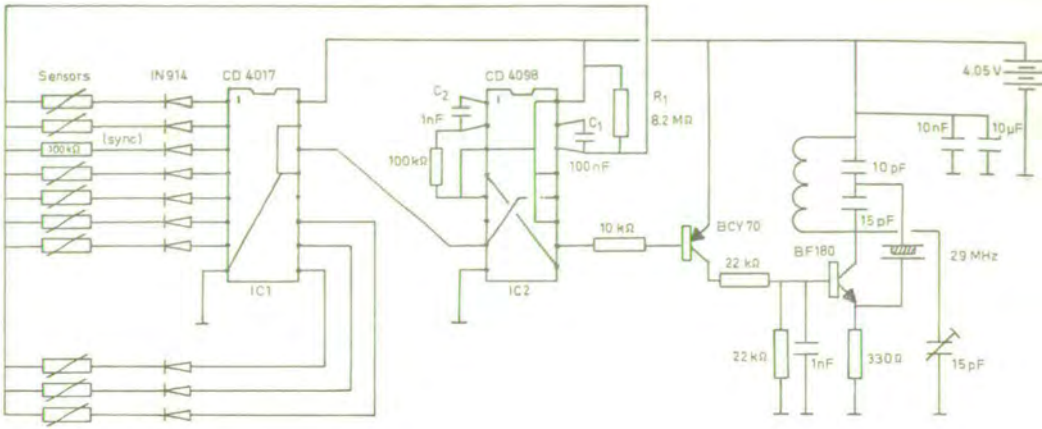


Fig. 6. The basic nine-channel transmitter.

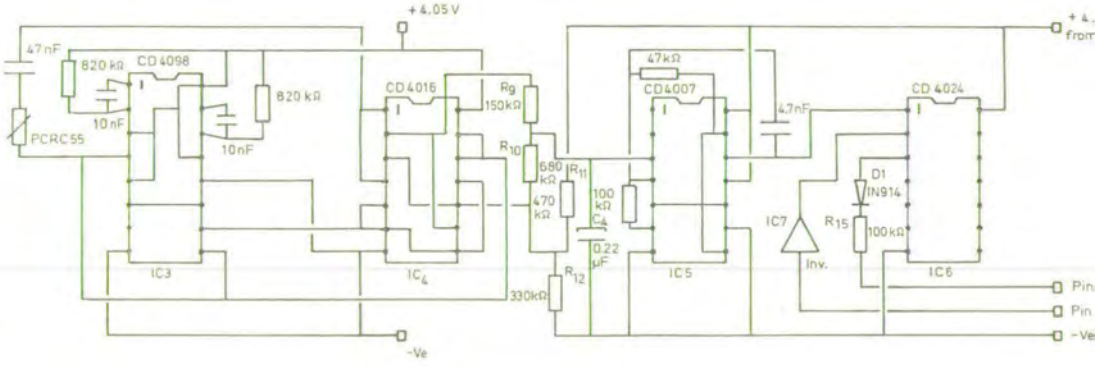


Fig. 7. The humidity sensor interface circuit.

Materials and Techniques

The volume of waterfowl eggs, namely 20–30 cm^3, is large for housing a modern biotelemetry transmitter. This allows the construction of the nine-channel transmitters by conventional techniques on miniature printed circuits. The circuit (fig. 6) of the basic transmitter, when built on a printed circuit board (43 × 25 mm), can be fitted into eggs $\geq$ 50 g. For smaller eggs, the circuit has been built on two smaller boards, or without a board. With the latter, the ICs are glued together in a stack

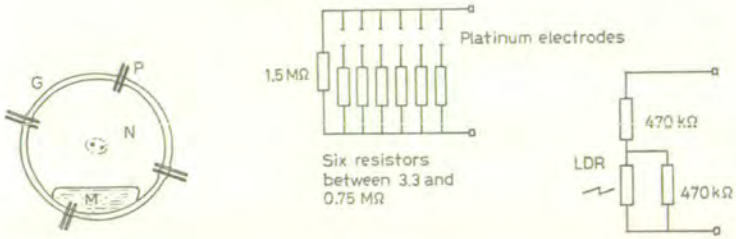


Fig. 8. The attitude sensor, associated resistor matrix and light sensor with limiting resistors. M = Mercury; N = nitrogen; P = platinum wire electrodes; G = glass.

with epoxy, the other components being fitted around them. This technique allows the construction of transmitters of as little as 5 g weight.

R_1 (fig. 6) allows the encoder to continue functioning should one or more of the sensors become open circuit. C_1 (fig. 6) should be a capacitor, or a combination of capacitors, which have a zero temperature coefficient over the temperature range in which the unit will be used. Frequencies around 29 MHz have been used, but others could be used with suitable changes to the RF oscillator.

The circuit (fig. 7) of the humidity sensor interface has been constructed using similar techniques to those for the encoder. The oscillator and synchronous rectifier (ICs 3 and 4) require a supply independent of the encoder. Resistors R_9 and R_{10} form the load for the sensor, their combination being chosen to give an output voltage in the control range of the VCO (IC5) over the required RH range (as shown 40–90%). R_{11} and R_{12} bias the VCO into its working range. C_4 smooths this control voltage. The divider (IC6) is held in the reset-zero state by a high on pin 2 derived from one of the outputs of IC1, in the encoder, via inverter IC7. D_1 isolates the divider from the encoder when the output is low. R_{15} feeds timing pin 14 IC2 (encoder) and ensures that any pulse generated for the humidity channel is longer than the synchronizing pulse.

The eggs were moulded from fibreglass using conventional techniques in a fibreglass mould taken from an egg of the species under study. A collar-joint formed from two interlocking Perspex rings fitted to the equator of the shell allows access to the electronics.

Sensors

Matched sets of six thermistors (type YSI 44015, Yellowstone Inst. Co., Ohio, or ITT GM105, ITT Thermistor Division, Taunton, England) were used in each egg. One was located alongside the humidity probe, the other five were embedded in the surface of the shell, four around the equator and one at the pointed pole.

A partially evacuated, hollow glass sphere (1.5 cm diameter) fitted with six pairs of platinum electrodes (0.5-mm diameter wire) equally spaced over its inner surface and containing a drop of mercury was used to sense the attitude of the egg (fig. 8). The drop of mercury is of such a size that it can only complete the circuit of one

pair of electrodes at a time. The sensor is positioned within the egg so that a pair of its electrodes provides a reference point for each of the thermistors. Each pair of electrodes switches a different value resistor into circuit. Another resistor in parallel with these and of higher value ensures that a circuit is maintained even if none of the electrodes is in the mercury (fig. 8).

A photo-resistor (ORP12 Mullard) was used to sense the presence or absence of the parent on the nest during daylight hours. Since the resistive span of these cells is high (10 kΩ–10 mΩ), a pair of resistors are used to limit the resistance change between light and dark (fig. 8).

A sulphonated polystyrene RH sensor (type PCRC 55, Phys-chem Research Corp., New York, N.Y.) was located in a compartment isolated from the other electronics by a plastic membrane. The fibreglass shell above this chamber was perforated with approximately 50 0.5-mm holes.

Transponder

This receives the pulses on 29 MHz from the probe transmitter re-transmitting them on 459 MHz. It has been designed (fig. 9) for minimum current drain thus giving prolonged operation in the field. The receiver is a single-conversion superhet using the ZN414 TRF IC as the IF amplifier. T3 is biased near to its threshold by the R_8 R_9 D1 combination; it conducts on the receipt of the 100-μsec pulses. T_4 and T_5 act as a saturating switch, T_5 modulating the transmitter. The transmitter oscillator runs continuously, supplied from the stabiliser T_6, ensuring clean modulation and frequency stability. The complete unit has been constructed on a printed circuit (300–90 mm) using a maximum ground technique, particular attention being paid to decoupling in the transmitter. The receiving antenna is a 1-metre length of wire wrapped around the nest, matched to 50 Ω by a ferrite bead transformer. A length of miniature coax cable runs between this and the transponder. The transmitting antenna is a conventional Yagi.

Receiver

A purpose-built double conversion superhet with two crystal-controlled oscillators has been used. This has IF frequencies of 29 MHz and 455 kHz. Different crystals, electronically switched by diode gates controlled by the data-logger, can be switched into the second oscillator allowing several transmissions to be automatically logged at programmed intervals. Data are logged on a standard Cristie (Stroud, England) CDL100 logger, fitted with ten timer-counters (fig. 5) and circuitry to log the date, time and experiment number.

Computer Analysis

After initial sorting, the data are checked to determine whether they are within the defined limits for each channel. If not, they are rejected. The length of the synchronizing pulse is then compared with its duration when the system was calibrated. Any deviation greater than $\pm$ 2 on the least significant digit is then extrapolated as a correction factor for each channel. This refinement is rarely needed if the timing capacitor (C_1) in the transmitter is correctly chosen, and the battery voltage remains stable.

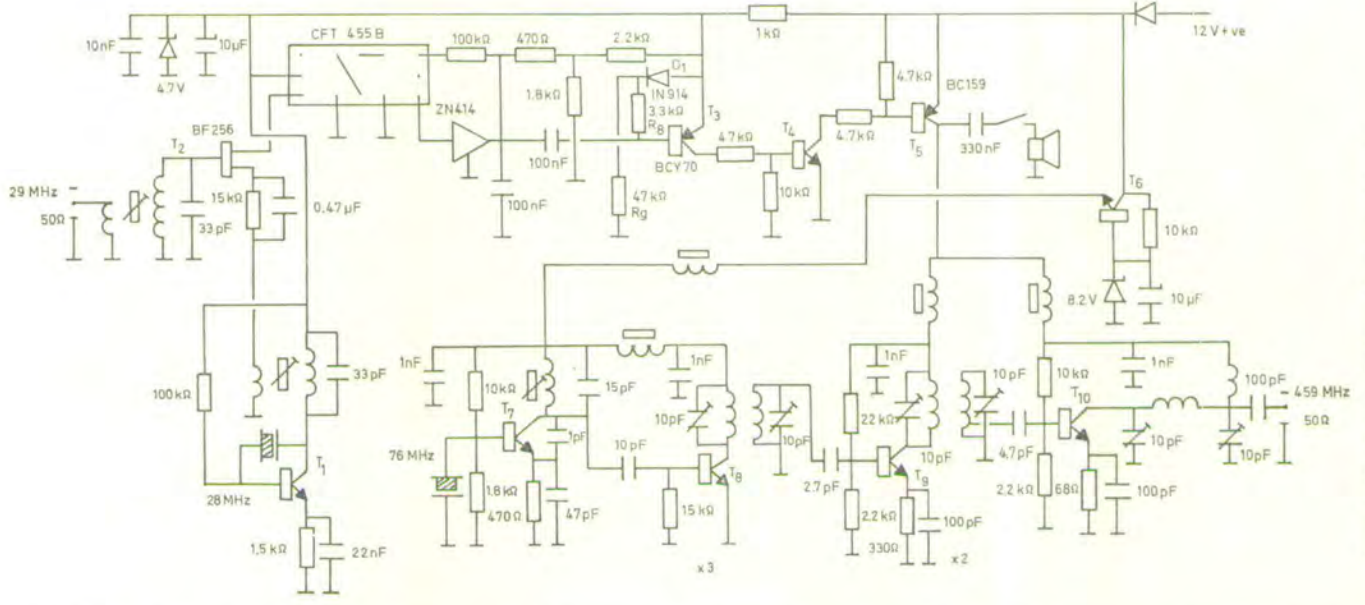


Fig. 9. The 29- to 459-MHz pulse transponder. All transistors are BF224 unless otherwise marked. Unmarked chokes are 2 turns on FX1115 ferrite bead.

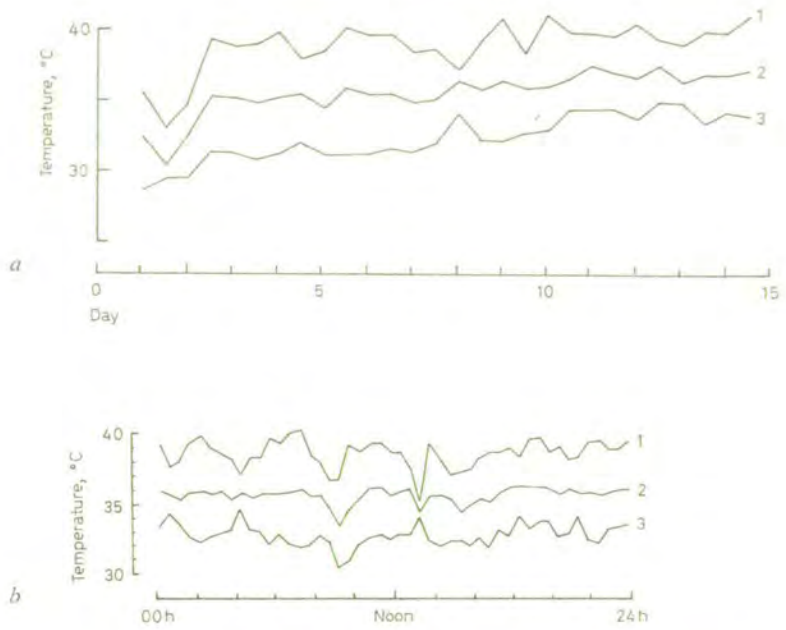


Fig. 10. Temperatures in the nest cup of the Greylag goose recorded during the first 15 days of incubation; sorted by the computer with reference to the attitude sensor (a), and after compilation into 'average day' (b). 1 = Temperature at the top of nest cup; 2 = mean temperature around sides of the sensing 'egg'; 3 = temperature at the bottom of the nest cup.

Applications and Results

Eggs ranging from 15 to 300 g have been constructed and used to monitor the nests of waterfowl, bantams and pigeons during the last two breeding seasons. There has been no evidence of rejection of the eggs from any of the nests studied.

An example of the temperature profile measured across a nest during the first 15 days of incubation is shown in figure 10a. This was plotted after computer sorting of the temperature measurements from each sensor with reference to the data from the attitude sensor. The initially low mean temperature can be attributed to the lower attentiveness of the parent and the initial dampness of the nest providing a greater heat sink during the first 2 days of incubation. The same data plotted on a 24-hour basis are

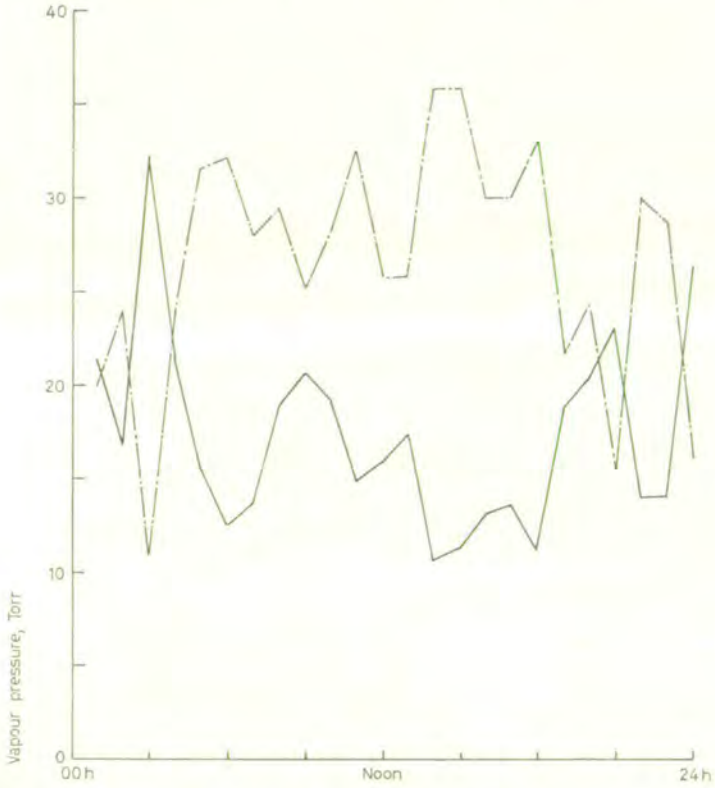


Fig. 11. The nest atmosphere absolute humidity plotted as 'average day' from data collected between days 9 and 21 of the incubation of the black swan. —— = VP nest atmosphere; – · – = gradient between nest atmosphere and egg shell membranes (calculated).

shown in figure 10b. The trend for a lower mean temperature during daylight is due to the parent leaving the nest. All of the species monitored have turned their eggs throughout day and night. The mean interval between turns appears to be species-dependent. In all instances eggs have been turned randomly between different positions. An example of the absolute humidity levels in the nest atmosphere, together with the calculated vapour pressure gradient between this and the incubating eggs, is shown in figure 11. This example was reconstructed by the computer from data recorded between days 9 and 21 of the incubation of a black swan.

Discussion

The telemetry system described here has proved reliable under the arduous conditions of use in the field. The digital approach to the decoding of the signals with automatic calibration drift compensation overcomes many of the problems of drift encountered with previous-generation systems, especially when used for long-term monitoring.

The technique would appear particularly useful for the study of thermoregulation, where it is necessary to monitor several temperatures simultaneously. The low component count and power requirement make it particularly suitable for implantation.

Although it has many advantages, the performance of a pulse-modulated system is inferior to that of a frequency-modulated system under noisy conditions. However, recording by digital logging rather than reconstruction of the original signal in analogue form (e.g. on chart) allows the selection of good frames of data from what otherwise looks like a useless signal. A single frame is often all that is required to obtain valid data when monitoring slowly changing parameters.

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References

1 KIMMICH, H. P.: Multichannel biotelemetry. Biotelemetry *2:* 207–255 (1975).

2 KOSSACK, C. W.: Incubation temperatures of Canada geese. J. Wildl. Mgmt *11:* 119–126 (1947).

3 SCHWARTZ, A.; WEAVER, J. D.; SCOTT, N. R., and CADE, T. J.: Measuring the temperature of eggs during incubation under captive falcons. J. Wildl. Mgmt *41:* 12–17 (1977).

4 VARNEY, J. R. and ELLIS, D. H.: Telemetering egg for use in incubation and nesting studies. J. Wildl. Mgmt *38:* 142–148 (1974).

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The microclimate of the nests of waterfowl

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A sophisticated radiotelemetry system was used to monitor continuously the temperature, relative humidity and the incidence of parental movement sufficient to admit light to, or turn the eggs in, the nest cup of (number of days recordings in parenthesis): Black Swan *Cygnus atratus* (68); Barnacle Goose *Brantal leucopris* (70); Whooper Swan *Cygnus cygnus* (28) and Greylag Goose *Anser anser* (26). The following features were common to all the nests: (1) a slow rise in the temperature of the nest cup with the onset of incubation; (2) a temperature gradient in the vertical plane of the nest cup throughout incubation; (3) a diurnal rhythm in the temperature and humidity of the nest cup, this being caused by bird behaviour rather than the ambient environment; (4) nesting materials dried out during incubation thereby accentuating the steepness of the diffusion gradient between a real egg and the atmosphere of the nest cup; (5) the eggs, which were turned between 0·59–0·95 times an hour, were rotated mainly around their long axes.

Although ornithologists have accumulated a large amount of information from field observations of brooding birds, there is only limited knowledge about the environment of the nest cup (Drent 1970, 1973, 1975). Even as regards temperature, in theory the most easily measured parameter, the paucity of information led Carey (1980) to conclude that 'the optimal temperatures for normal embryo development and maximum hatchability have not been documented extensively for embryos of wild species'. Methods used for field studies of nest cup temperatures were based initially on thermographs contained in dummy eggs in the cup, or thermal sensors (attached to the inside of a nest cup or to real or artificial eggs) connected by leads to recording apparatus (commonly paper charts) in hides situated near to the nest. Not only did these sensing and recording methods not lend themselves to long-term recording or detailed analysis, the requirement for hides meant that artifacts due to human intrusion could not be discounted. Although some of these problems were overcome by using radiotelemetry (Schwartz *et al.* 1977), the use of paper charts curtailed detailed analysis. Nevertheless these workers did improve the hatchability of artificially incubated eggs of Prairie Falcons *Falco mexicanus* by setting the temperature of the incubator at a lower value than that used previously.

The poultry industry have achieved commercially acceptable levels of hatchability by applying empirical observations made over hundreds of years (Landauer 1967) and probably also by applying unwittingly strong selective pressures on domesticated species of birds so that their eggs hatch in an incubator. Apart from the temperature of the incubator, a certain humidity in the incubator atmosphere (Lundy 1969) and, for as yet poorly defined reasons, occasional turning (Robertson 1961) are required for the successful artificial incubation of the eggs of domesticated species of bird. As yet radiotelemetry systems have not been used to monitor continuously these last two features. Egg turning has been studied mainly by recording changes in the location of marks on eggshells in nests from which the

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but, once a plateau had been achieved (Fig. 3), there was little oscillation. This was in marked contrast to the temperature found in the nest cup. The period (15–25 days) of maximum temperatures with minimum gradients began at the time when the ambient shade temperatures were highest (Fig. 3). In general, however, major oscillations in the temperature recorded by the monitoring device coincided with

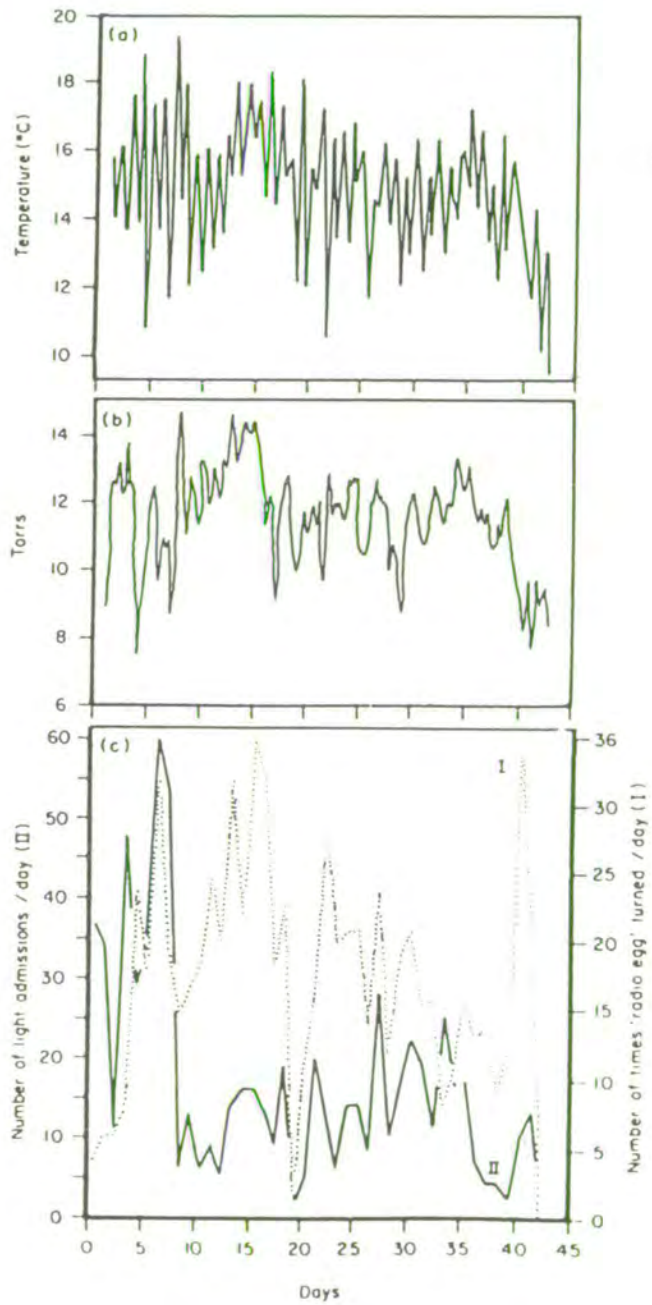

FIGURE 4 (continued overleaf)

parent was driven off (Drent 1970). Loss in the weight of eggs in a clutch (Drent 1970) or gain in weight of an egg hygrometer (i.e., an egg of the species under study filled with dry silica gel) have been used to calculate an average value for nest humidity (Rahn, Ackerman & Paganelli 1977) and the extent of nest cup ventilation (Burke 1925; Chattock 1925) that would ensure an egg lost an optimal amount of water during incubation, about 16–18% of its initial mass (Ar & Rahn 1980). Thus a radiotelemetry system would need to monitor not only nest cup humidity but also events that contribute to ventilation.

The various factors noted above influenced the design and construction of a radiotelemetry system which we designed for the routine monitoring of the nest cup environment (Howey, Board & Kear 1977). The 'radio egg' (Fig. 1) transmits data which are decoded and stored on magnetic tapes (Fig. 2) in a form suitable for computer analysis. It needs to be stressed that, through being of the same size, shape and weight as an average egg in a clutch under study and through having batteries sufficient to maintain transmission throughout the incubation period, our 'radio egg' caused little if any disturbance in marked contrast to systems that depend upon leads

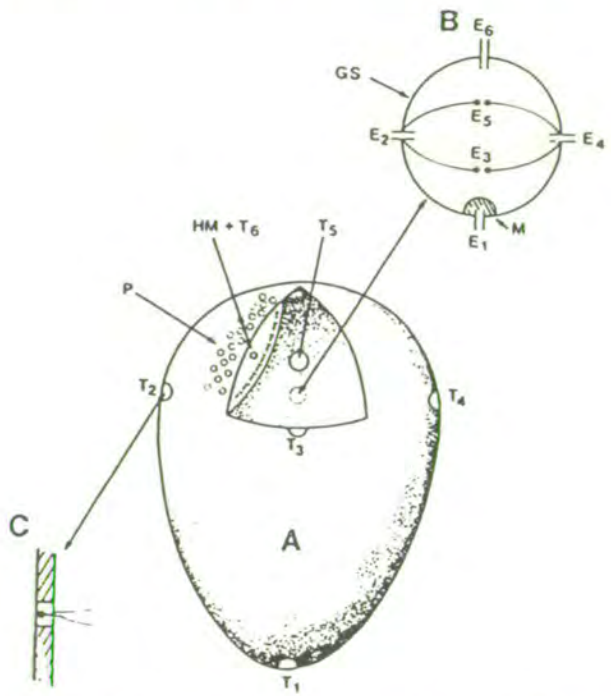

FIGURE 1. Stylized drawings of the monitoring egg (A) and attitude sensor (B). Five thermistors (T_1–T_5) were cemented (shown in C) into the fibreglass shell so that T_1 was at the pointed pole of the shell and T_2, T_3, T_4 & T_5 were spaced equidistantly around the equator. A sixth thermistor (T_6) and a relative humidity probe (HM) were contained in a compartment at the broad end of the shell, the compartment being formed by a diaphragm cemented to the inner surface of the fibreglass shell. The shell overlying HM was perforated (P) so that rapid equilibration occurred between the atmosphere of the chamber and the atmosphere surrounding the fibreglass shell. The attitude sensor (B) was a hollow sphere (GS) filled with dinitrogen and fitted with six pairs of platinum wire. Pairs E_1, E_2, E_4 & E_6 were on a circumference and pairs E_2, E_3, E_4 & E_5 on a diametrically opposing one. A bead of mercury (M) completed the circuit in a pair of patinum wires. The attitude sensor was located at the centre of the monitoring egg so that each pair of platinum wires was on the same radius as a thermistor, E_1 with T_1, E_2 with T_2 *et seq.* A radio transmitter, ancillary equipment and batteries sufficient to sustain transmission throughout incubation were fitted into the egg. The completed egg was given gravimetric symmetry with lead shot cemented to the inside of the shell.

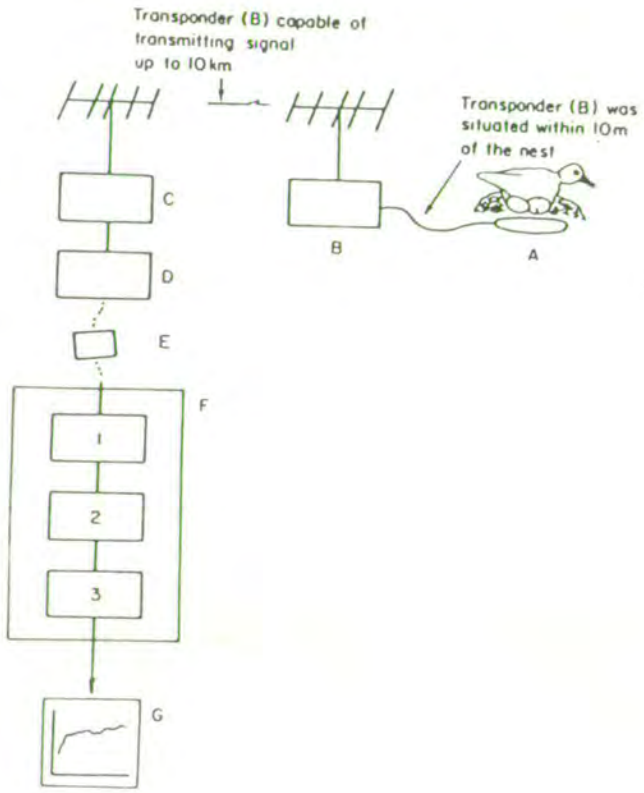

FIGURE 2. A schematic representation of the system used to monitor and analyse the microclimate of the nests of waterfowl. A, nest containing 'radio egg' and encircled with the aerial from the transponder (B) which transmitted signals back to a radio receiver (C) & decoder (D) in a laboratory. The signal from the decoder was recorded in digital form on a magnetic tape (E). The information from the magnetic tape was fed into a main frame computer (F). The data were sorted (F_1) to eliminate frames of information containing errors (for details of sorting methods, see Howey *et al.* 1977) and then calibrated and converted into appropriate units (F_2) for analysis (F_3) of the entire period or an 'average day' of incubation. The results were printed out in graphical form (G).

for transmission of information from the nest. This paper presents analyses of data (amounting to more than $3{\cdot}3 \times 10^5$ individual recordings) obtained during 174 days of continuous monitoring of the nests of captive Black Swans *Cygnus atratus*, Barnacle Geese *Branta leucopsis* and a Whooper Swan *Cygnus cygnus*. The birds nested in large enclosures at the Wildfowl Trust, Slimbridge, England, where they were fed twice daily at about 0830 and 1600 h. The Black Swans were particularly revealing, because they nest in almost every month of the year in the UK (Kear & Murton 1976) and are the only swans in which the sexes share incubation duties (Kear 1972), the male sitting for 5 or 6 hs around mid-day. The Whooper Swan was chosen because only the female incubates (Kear 1970), and the Barnacle Goose because large numbers were available at the time. Additional observations are presented on the nest of a captive Greylag Goose *Anser anser* where, as in the Barnacle Goose, the female alone incubates. The species differ in one other obvious feature: Black Swan nests contain very little feather down, Whooper Swan nests a little more, while the goose nests hold a relatively large quantity that is pulled over the eggs when the female leaves.

MATERIALS AND METHODS

THE MONITORING DEVICE

A fibre glass 'shell' was made in a mould of an average egg (size and shape) of each species studied, and various sensors were fitted inside (Fig. 1). Through having no gravimetric bias or external leads, the 'radio egg' could be freely moved by the parent. A weather station, designed on similar principles, was used to monitor ambient shade temperature, ground temperature (thermistor inserted 1 cm in soil), light intensity, relative humidity (sensor fitted with a thermistor) and temperature in the nesting material. The weather station was situated about 10 m from the nest.

Our 'radio egg' does not provide information about conditions present in a real egg; as shown by Schwartz *et al.* (1977), the temperature within an egg in a clutch before the embryos produce heat can be monitored with a dummy egg filled with liquid paraffin, a temperature sensor and a simple radio transmitter. Our egg gave a record of the microclimate surrounding a real egg, the number of times that the brooding bird caused eggs to be moved through at least 45°, and the number of times that light was admitted to the nest cup. The use of the 'radio egg' with chart recorders provided information about the duration of periods that the birds were off the nest.

FIELD STUDIES

The 'radio egg' was placed in the nest along with the eggs already there. The transmitted signal was picked up by an aerial hidden in the nest at ground level and relayed by a nearby transponder to a radio receiver in a laboratory. The radio signal was decoded (details given in Howey *et al.* 1977) and the data recorded on paper charts (example given in Fig. 8) or stored on magnetic tapes ready for analysis. To investigate the microclimate of a nest cup with paper chart recordings, it was necessary to transfer data from charts to punched cards for computer analysis. As it took one working day to transfer 24 h of records from charts to tapes, this cumbersome system was unsuitable for routine use.

COMPUTER ANALYSIS

Programmes were devised for the following: (a) to sort raw data (a frame—the recordings taken at any one time from all nine sensors—was discarded if any reading was outside predefined limits. On some occasions the relationship of the 'radio egg' to the aerial of the transponder was such that incomplete or distorted frames were recorded; on some occasions also interference by other radio systems caused such faults). (Fig. 2); (b) to calibrate the information from sensors subject to drift, *viz.* the thermistors and humidity meter; (c) to relate the recordings from the thermistors to their position in the nest cup; and (d) to plot the records for the entire incubation period or for an average day. Chatfield's (1975) exponential smoothing technique was used to prepare graphs for the entire observation period. The recordings obtained with the relative humidity meter were used to calculate the absolute vapour pressure of the nest cup atmosphere and the gradient (in torrs) existing across the shell of a real egg and the nest atmosphere.

RESULTS

Table 1 lists for each bird studied the duration of observation periods, the quantity of data available for analysis and the intervals between recordings from all nine sensors in a 'radio egg'. All data were obtained from nests in which eggs eventually hatched. The major findings during continuous monitoring are summarized below and in Table 1.

1. A slow increase in the nest temperature over several days after the onset of incubation.
2. A temperature gradient obtained vertically through the nest cup and the underlying nest material.
3. The eggs were turned, on average, 0·5–0·95 times per hour and in almost all cases around the long axis.
4. The nest material dried out during incubation.
5. Convection and parental movement contributed to moisture loss.
6. The temperatures recorded in the nest cup were lower than those used in commercial incubators for hatching the eggs of domestic hens.

ENTIRE OBSERVATION PERIODS

The recordings made during the 42 days of observation of a Black Swan nest, presented in Figures 3, 4 and 5, will be discussed in detail. The two eggs present at the start on 7 October 1977 were cold to the touch, the 'radio egg' recorded a temperature of about 20°C (ambient temperature, 13·5–17·0°C) and there was only a slight temperature gradient in the vertical plane of what at that time was a pile of vegetation containing a small amount of down and the partially buried eggs. One parent was always near or on the nest in the following three days during which the five-egg clutch was completed. Only for this pair did monitoring begin before the onset of incubation. Continuous incubation started on Day *3* of observation when the last egg was laid. A deep cup was formed in the nest mound and the temperatures recorded by the 'radio egg' rose slowly to a peak on Day *15*, 12 days after continuous incubation began. A similar slow temperature rise was noted in a Barnacle Goose nest (Fig. 6). The temperature of the Black Swan's nesting material rose slowly also

TABLE 1

The waterfowl nests used in the incubation study, the number of days of continuous observation and the quantity of data available for analysis

	Days of observation *n*	Recordings available from each sensor for analysis[1] *n*	Average period (min) between valid observations
Black Swans			
1[2]	21	6306[3]	4·8
2	4	1704	3·4
3	43	12494	5·0
Barnacle Geese			
1	20	4245	6·8
2	25	10349	3·5
3	25	[4]	Continuous recording
Greylag Goose			
1	26	[4]	Continuous recording
Whooper Swan			
1	10	2385	6·0
Totals	174	37483 × 9 senors = 337347	

Notes: [1]After stringent sorting of the recorded observations, about 75% were available for analysis. [2]The numbers refer to nests of different pairs. [3]Recordings made directly onto magnetic tape in a form suitable for computer analysis. [4]The recordings stored on paper charts were transferred to punched cards for computer analysis.

TABLE 2

A summary of the average values of the absolute vapour pressure of the waterfowl nest cup, the vapour pressure gradient between the egg and the nest cup atmosphere and the average number of times eggs were turned per hour

	Average absolute vapour pressure of nest cup (torrs)	Average absolute vapour pressure of ambient air (torrs)	Gradient in vapour pressure between egg and nest cup	Number of times an egg was turned
Black Swan				
1[1]	20·25	—[2]	21·56	0·59
2	28·75	10·95	21·13	0·89
3	18·25	11·74	23·19	0·77
Barnacle Geese				
1	17·0	7·89	14·75	0·50
2	18·0	10·95	15·00	0·95
Whooper Swan	32·89	—	13·67	0·60

Notes: [1]Numbers refer to individual nests. [2] No recordings made.

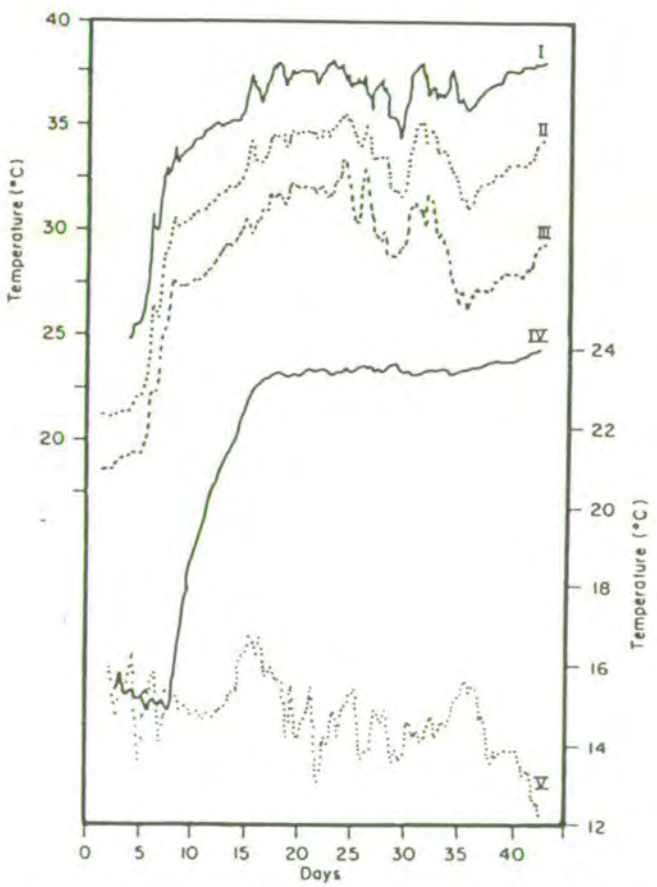

FIGURE 3. The temperatures in the nest cup (I, II, III), nesting materials (IV) and ambient atmosphere (V) in the 43 days' observation/incubation of a Black Swan (No. 3, Table 1) at the Wildfowl Trust, Slimbridge, Glos. Observations began on 7 October 1977. I, temperature recorded by thermistor (Fig. 1) at or near the top of 'radio egg' II, mean temperatures recorded from the thermistors located at some place between the breast of the brooding parent (I) and the floor of the nest cup (III). IV, thermistor inserted in nesting material immediately below floor of the nest cup. All data exponentially smoothed.

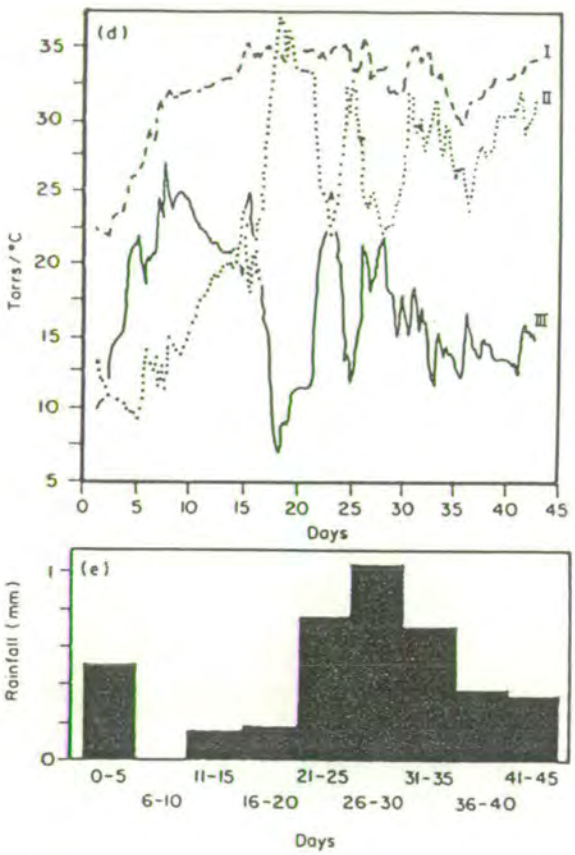

FIGURE 4 (a) Records of ambient shade temperatures, not exponentially smoothed; (b) absolute vapour pressure of bulk atmosphere; (c) bird activity : I, incidence of turning of 'radio egg', II, incidence of light admission to the nest cup; (d) temperature (I) and absolute vapour pressure (III) of the nest cup atmosphere and the diffusion gradient (II) between the real egg and nest cup atmosphere, and (e) rainfall. Records obtained during 43 days of observation of the nest of a Black Swan (No.3, Table 1).

bird activity that admitted light to the nest cup. Such events were most common during the early stages of incubation when the nest cup temperature was rising slowly. When the incidence of light admission to the nest cup was used as an index of bird activity, it appeared that the bird (or birds) sat especially tightly during the last two days of incubation (Fig. 4). The upward trend in the temperature of then nest cup at that time (when the lowest ambient shade temperatures were recorded) was presumably a consequence of the increased level of attentiveness as well as heat production by the embryos.

Bird behaviour leading to egg-turning appeared to determine the extent of the thermal gradient in the vertical plane of the nest cup (Fig. 3). Thus the gradient was minimal during periods when the incidence of egg-turning was high and *vice versa*. The large number of times that the monitoring egg was turned at the end of incubation reflects presumably the activity of the cygnets, both during and after hatching, as well as increased parental activity (Figs 3 & 4). This was a common feature in all nests in which monitoring was continued throughout incubation.

Although the absolute water vapour pressure of the nest cup increased as the temperature in the nest cup rose with the onset of incubation (Fig. 4), it did not

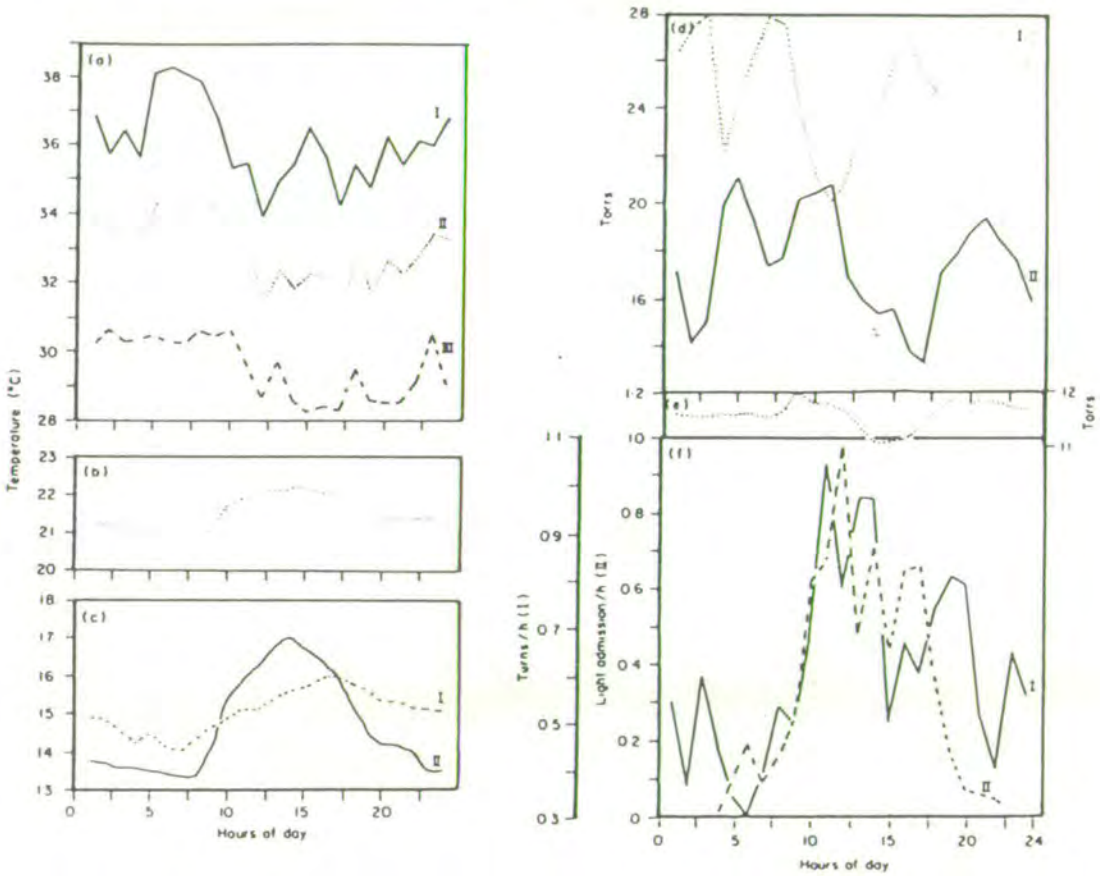

FIGURE 5. An 'average day' of incubation of a Black Swan (No. 3, Table 1). (a) Temperatures at the top (I), middle (II) and bottom (III) of 'radio egg'; (b) temperature in nesting material (thermistor situated immediately below the floor of the nest cup), (c) ambient shade (I) and ground temperature—thermistor inserted (1 cm in soil)—(II); (d) absolute vapour pressure of nest cup atmosphere (II), and calculated diffusion gradient between real egg and nest cup atmosphere (I); (e) absolute vapour pressure of ambient atmosphere, and (f) number of times 'radio egg' turned per hour (I) and number of light admissions to nest cup per hour (II).

follow the temperature profile from the seventh day onwards. This was taken as evidence that brooding caused the mound of nesting material to dry out and thereby accentuate the vapour pressure gradient between the real eggs and the nest cup atmosphere. These trends were common to all the nests included in this study. These general trends were interrupted by large oscillations between the 15th and 30th days of incubation (Fig. 4). The first major trough in the record of the vapour pressure in the nest cup coincided with the lowest incidences of light admission and egg-turning, and came at the end of a 15-day period during which only 1·1 mm of rain fell near the nest. The peak following the trough occurred during a period of rain (Fig. 4) and at a time of active egg-turning. Such turning tended to decline thereafter and the general tendency for the vapour pressure of the nest cup atmosphere to diminish also appeared to be related to an increase in bird behaviour resulting in light admission to the cup. The slight increase in vapour pressure during the last two days of incubation was attributed to water loss from pipped eggs, the lungs of the cygnets and the moist down of newly-hatched cygnets.

AN 'AVERAGE DAY' OF INCUBATION

A diurnal rhythm was evident in the graphs obtained by averaging all the recordings made throughout an observation period to give an 'average day', the bird(s) themselves being the principal cause of the rhythm. With the Black Swan (Fig. 5) and Barnacle Goose (Fig. 6), there were pronounced peaks and troughs in the temperatures in the nest cup during an average 24 h period, the first trough occurring between 0230 and 0600 h. With the Black Swan (Fig. 5), it occurred at 0230 h, at which time active egg-turning was a feature. The temperature at the top of the egg cup attained the highest level immediately following the first trough, but the temperature gradient in the vertical plane of the nest was at its maximum value for the 24 h period, presumably because of infrequent egg-turning. The Barnacle Goose (Fig. 6) showed little behaviour resulting in light admission to the nest cup during the initial period of low temperatures (at 0600 h) which occurred about two hours after sunrise. The lowest temperatures in the nest cup of the Black Swan (Fig. 5) were recorded when the incidence of light admission to the nest cup and egg-turning were at their maxima (at about 1100 h). This was when the male took over incubation from his mate. In this species, the second major peak in nest temperature occurred at 1500 h, at which time the brooding bird was relatively quiescent and the ambient shade temperature as well as that of the nest material were at their maximal values for the day. The trough following this peak was notable for a period of a high incidence of light admission to the nest cup, due most probably to the birds again swapping duties. Thereafter minor peaks and troughs in the temperature recordings were correlated with egg-turning. Indeed the Black Swans gave no evidence of an interaction between the temperatures of the nest cup and the ambient environment. In the Barnacle Goose (Fig. 6), where only the female incubated, the lowest temperatures in the nest cup were recorded at 1700 h, the time at which the bird left for the longest period (Fig. 11) to feed. In addition, the oscillations in the daily temperatures of the nest cups of the Barnacle Geese (*viz.* Fig. 6) were less than those in the swan nests studied, probably because the large amount of down in goose nests insulated the eggs better.

Three major peaks occurred in the absolute water vapour content of the nest cup of the Black Swan in a 24 h period (Fig. 5). The first occurred at 0500 h following the period (at 0230 h) of low temperatures in the nest cup atmosphere. The second occurred at about 1100 h at a time of maximal bird activity leading to a high incidence of light admission to the nest cup and egg-turning, when we assumed that the mates were changing places, and the third (at about 2100 h) followed a period during which egg-turning was frequent.

The major trough in the water vapour content of the nest atmosphere—and hence the longest period for potential water loss from the real eggs—occurred in the afternoon in the Black Swan's nest (Fig. 5) at a time when the incidence of light admission to the cup was relatively high and incubation was probably by the male. Indeed the bird's behaviour leading to light admission is almost certainly the major cause of nest ventilation. Only one major peak occurred in the water vapour pressure in the nest cup of the Barnacle Goose (Fig. 6), in which only the female incubated. This coincided with a high incidence of egg-turning which occurred at 1800 h following feeding. The increase in water vapour content of the atmosphere of the nest cup between 1000–1830 h was probably due to water being brought to the nest on the bird's plumage. This was the only bird in our studies that nested on an island and, judging from the results in Figure 11, it left the nest for relatively short periods before a major absence for feeding. Probably water was brought to the nest on the bird's plumage after each absence and this could account for the modest trough in the vapour pressure of the nest cup during daylight hours.

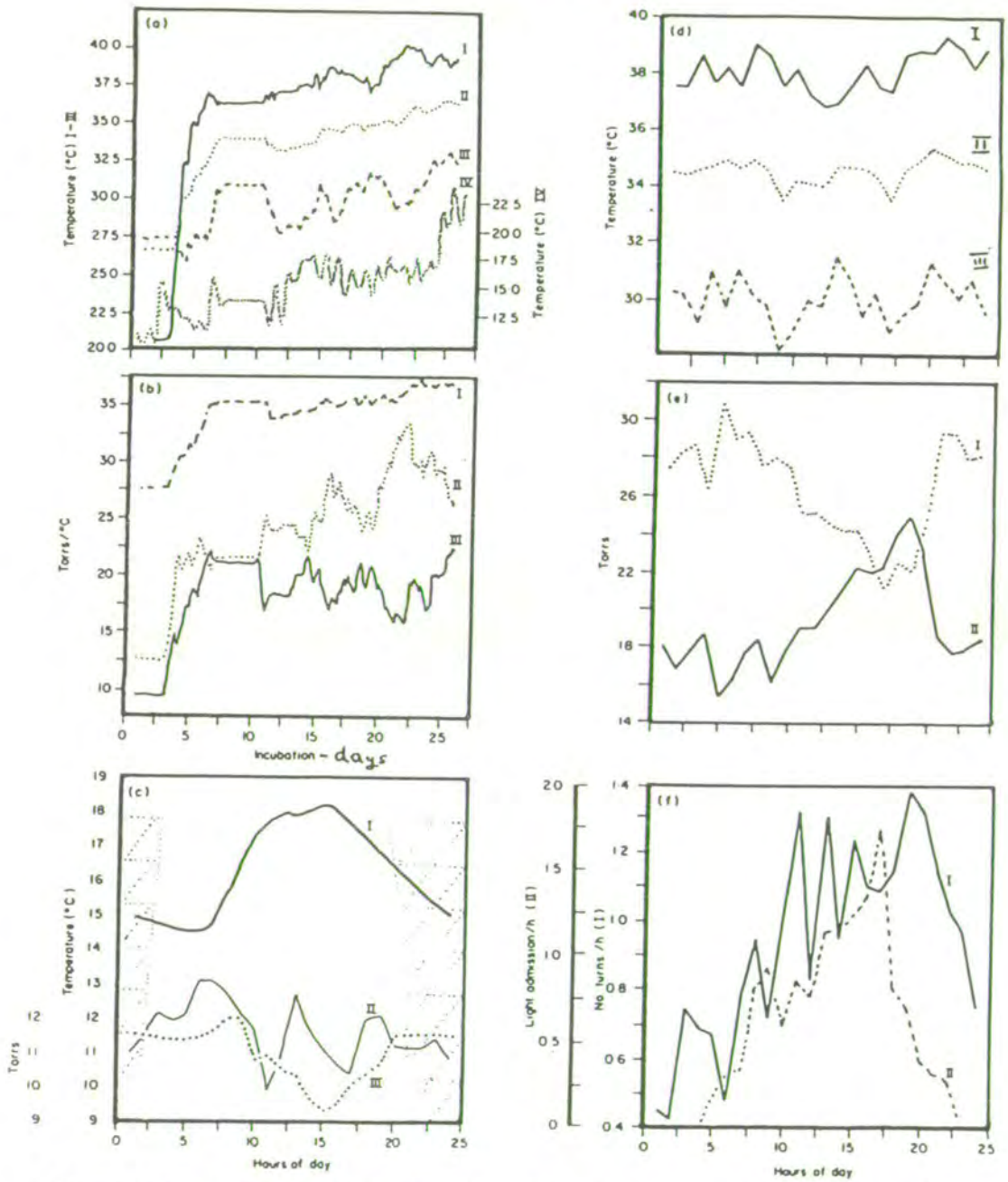

FIGURE 6. The recordings made during observations of the nest of a Barnacle Goose (No. 2, Table 1). Entire period of incubation. (a) The temperature recorded at the top (I), middle (II) and bottom (III) of the 'radio egg' and ambient shade temperature (IV), and (b) average nesting temperature (I), calculated diffusion gradient between real egg and nest cup atmosphere (II), and absolute vapour pressure of nest cup atmosphere (III). An 'average day', hatching—night time. (c) ambient shade temperature (I), soil temperature (II), and absolute vapour pressure of the ambient atmosphere (III); (d) temperatures at the top (I), middle (II) and bottom (III) of 'radio egg'; (e) the calculated water diffusion gradient between the real egg and the nest cup atmosphere (I) and the absolute water vapour pressure of the nest cup atmosphere (II), and (f) the number of times per hour that the 'radio egg' was turned and the incidence of light admission to the nest cup (II).

Although water vapour was lost from the nest cup of the Black Swan (Fig. 5) mainly during periods of bird activity, convection of water vapour through the nesting material probably contributed also. Thus the major trough in the vapour pressure of the nest cup occurred not only at a time when the male was sitting, but also when temperatures of both the nesting materials and the ambient environment were highest. Indeed the average values (Table 3) for the absolute water vapour pressure of the nest cup atmosphere, the gradients between it and a real egg and between it and the ambient environment, would have ensured water loss by diffusion from all the nests we studied.

The daily changes in the temperature and vapour pressure of the nest cup of the Whooper Swan (Fig. 7) were similar to those found in the nests of the Black Swan (Fig. 5) and Barnacle Goose (Fig. 6).

EGG-TURNING AND LIGHT ADMISSION

As mentioned, the incidence of egg-turning changed during incubation (Figs 4, 6) and throughout an 'average day' (Figs 5, 6, 7). The observations made with the 'radio

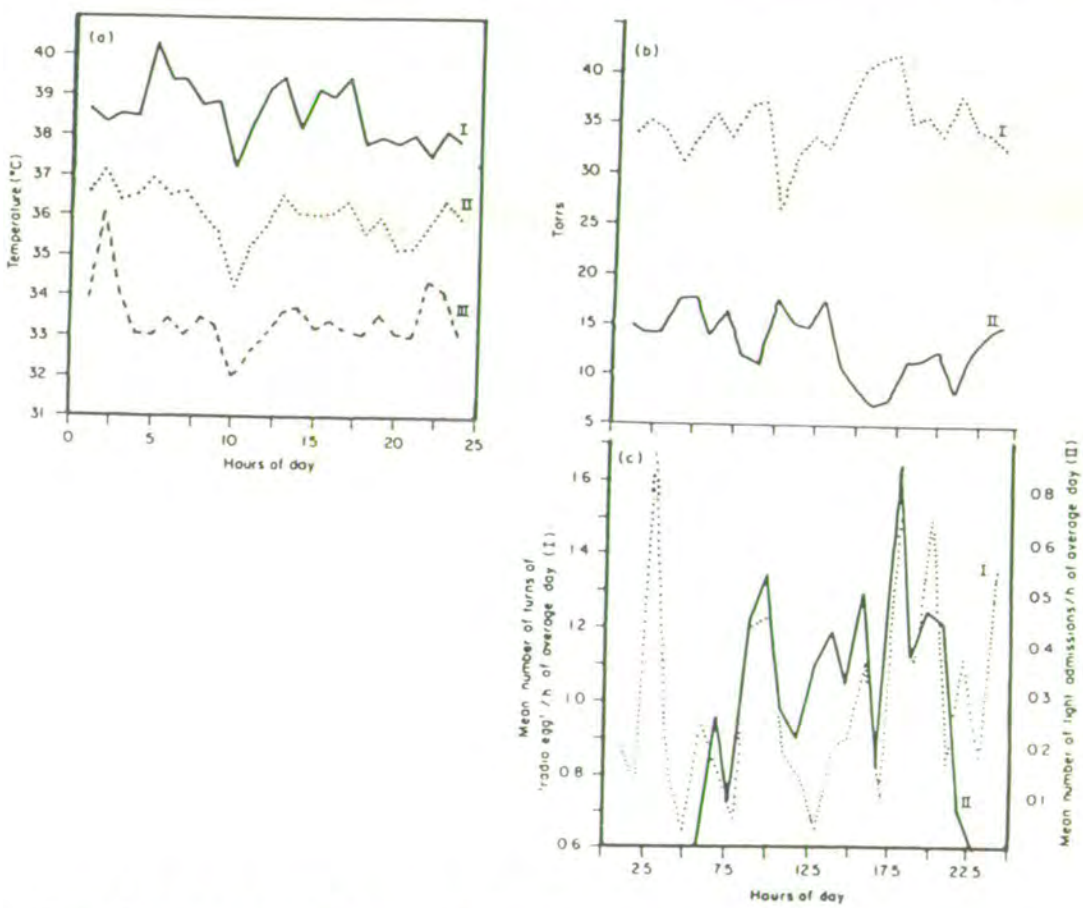

FIGURE 7. An 'average day's' incubation of a Whooper Swan (Table 1). (a) Temperatures recorded from the top (I), middle (II) and bottom (III) of 'radio egg' (b) the calculated water vapour diffusion gradient (I) and the absolute vapour pressure (II) of the atmosphere of the nest cup, and (c) the number of times per hour that the 'radio egg' was turned and the incidence of light admission to the nest cup (II).

egg' and magnetic tape recordings suggest (Table 3) that the position of a waterfowl egg changes on average less than once an hour. Moreover in the majority of cases, the 'radio egg' was turned around its long axis.

Although the scope offered for detailed analysis of the nest cup microclimate by the 'radio egg' and magnetic tape recordings is vastly greater than that of all previous methods of monitoring, we still had to resort to paper chart recordings for certain analyses. Thus Figure 8 covers 7 h in the 26-day incubation period of a Greylag Goose and shows clearly the number of times that the 'radio egg' was turned and the number that the bird rose sufficiently to admit light to the nest cup or left the nest. The influence of the last two events on the temperature and relative humidity of the nest cup is evident also. Moreover the paper charts allowed us to investigate the relationship between bird behaviour causing light admission to the nest cup and egg-

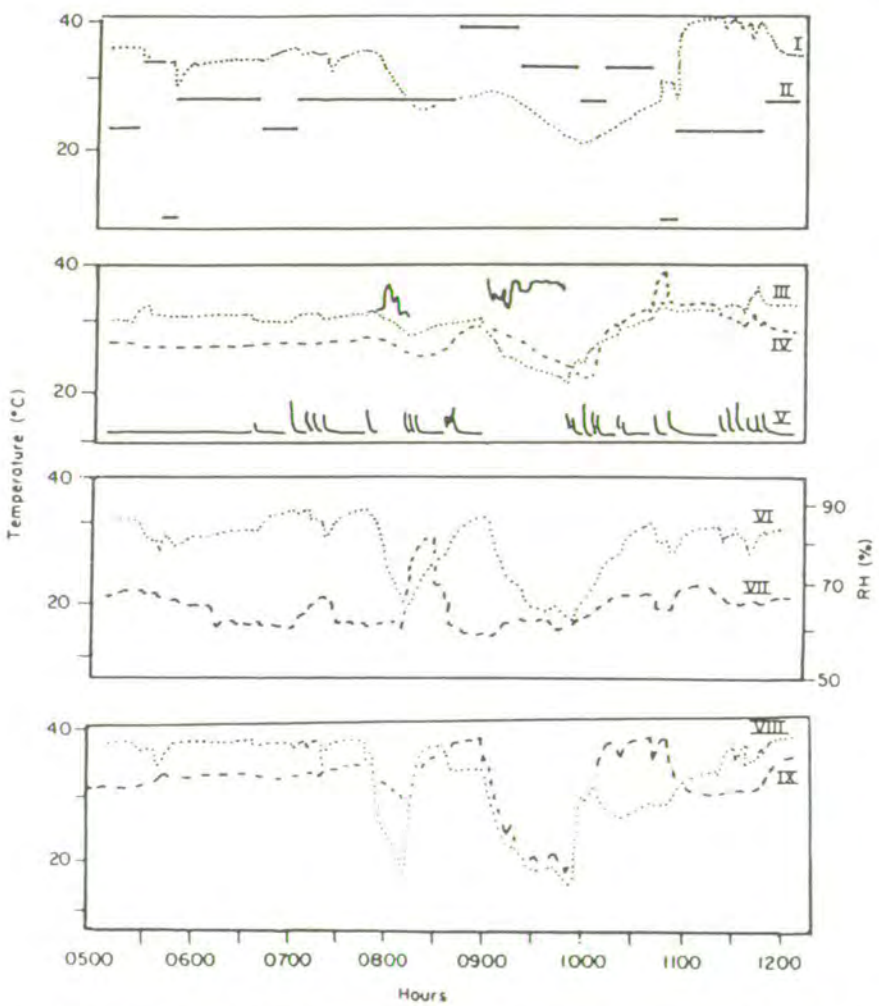

FIGURE 8. A composite trace of the paper charts taken from four chart-recorders during 7 hs of observations of the nest of a Greylag Goose (Table 1). I, III, IV, VI, VIII and IX, thermistors in 'radio egg', II, attitude sensor; V, light sensitive cell, and VII, relative humidity meter. Locations of sensors shown in Figure 1.

turning, and to establish how long birds spent away from the nest. There was a poor correlation between light admission and egg-turning in the Greylag and Barnacle Geese (Fig. 9). In the latter, the pattern obtained from analysis of paper charts on the frequency with which the bird stood up and admitted light to the cup (Fig. 9) was very similar to that given by computer analysis of recordings on magnetic tapes (Fig. 6). Moreover Figure 9 demonstrates that, when judged by the incidence of light admission to the nest cup, the Greylag Goose was relatively more active than the Barnacle Goose. This trend was evident also in the time that the two birds spent away from the nest throughout the incubation period (Fig. 10). However, the periodicity of light admission to the nest cups varied appreciably between the different birds studied. Two major daily peaks occurred in the Black Swan (Fig. 5), probably associated with the parents swapping duties, but only one major peak occurred in the Greylag Goose. Although the data were limited (Fig. 7), there were two peaks in the Whooper Swan records also, even though only one bird incubated. When time away from the nest was considered, the Barnacle Goose had a short period during the morning followed by a long period in the late afternoon (Fig. 11). Although only one major peak was evident (Fig. 11) in the sum of the time that the Greylag Goose spent away from the nest throughout incubation, analysis of consecutive five-day periods revealed that the three minor peaks at the top of the major one were due to the bird leaving the nest later during successive days.

DISCUSSION

The use of a sophisticated 'radio egg' to monitor continuously many parameters of the nest cups of the waterfowl included in this study has directed attention at (a) the relatively low incidence of egg turning—0·59–0·95 turns per hour, (b) the slow

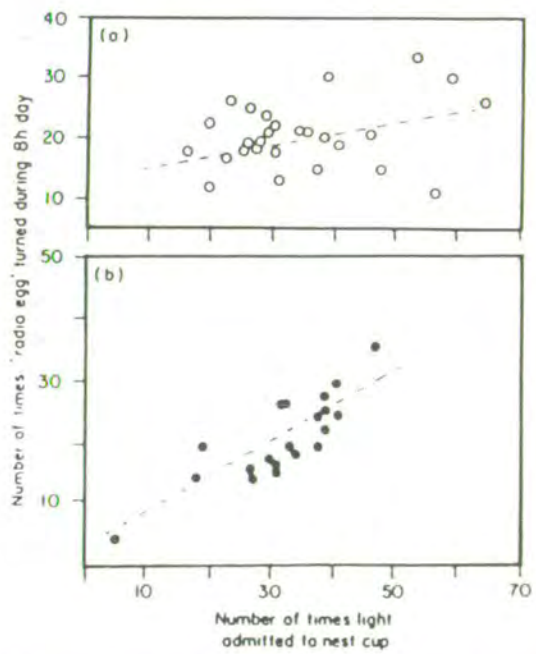

FIGURE 9. Comparison of the number of times the 'radio egg' was turned and light was admitted to nest cup. (a) Greyleg Goose, and (b) Barnacle Goose (No. 3, Table 1). These Figures were compiled from paper-chart recordings of the type exemplified in Figure 8.

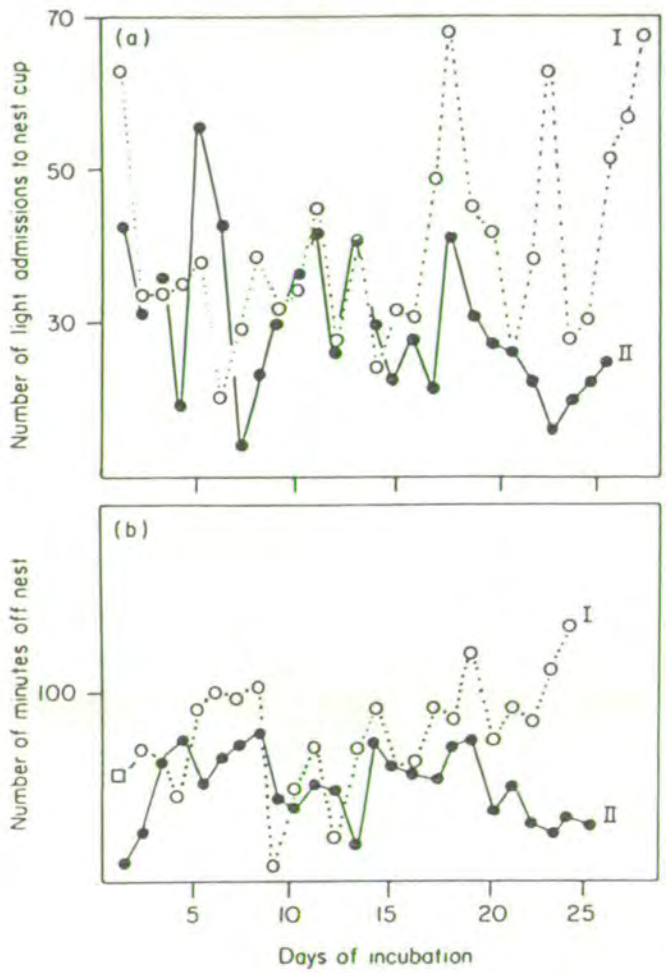

FIGURE 10. (a) The number of times per day that light was admitted to the nest cups of a Greylag Goose (I) and a Barnacle Goose No. 3 (II). (b) The total periods per day that the Greylag Goose (I) and the Barnacle Goose No. 3 (II) was away from the nest. These Figures were composed from information recorded on charts such as those illustrated in Figure 8. Details of incubation times are given in Table 1.

increase in the nest temperature after the onset of incubation, (c) the drying out of the nest materials, and (d) the role of the parents in nest ventilation.

It has been customary to consider the thermal environment of the nest cup in the narrow context of the temperature that supports embryo development. We are of the opinion that the maintenance of viability in the blastoderm of the first-laid egg and induction of embryo development ought to be considered also. Our observations (Figs 4, 5, 6) may be of particular relevance in this context. Temperatures in the nest cup of the Black Swan were always greater than ambient, even before incubation began, and it would seem reasonable to assume that the nest material acted as a lagging thereby ensuring the well-being of the embryo when the parents were not sitting. A slow rise in the temperature of the nest cup with the onset of incubation was a feature common to all four waterfowl species studied. This is probably in marked contrast to the situation in artificial incubators in which the eggs are brought up to

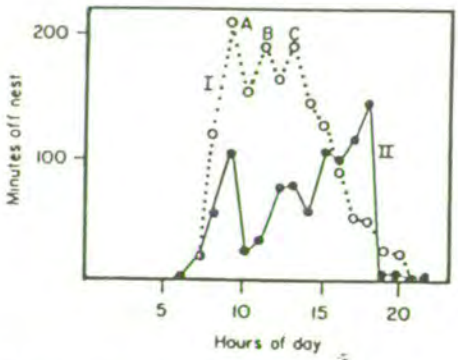

FIGURE 11. Total periods of absence from nest by Greylag Goose (I) and Barnacle Goose No. 3 (II), shown against each hour of the day for the entire period of incubation (26 days). The data were taken from charts of the type illustrated in Figure 8. Further analysis of the charts obtained with the Greylag Goose (I) showed that peak *a* was due mainly to bird activity in 0–5 days of incubation, *b* to activity in the 6–15 days, and *c* activity in the 11–20 days of incubation. Details about the two birds are given in Table 1.

incubation temperature in a relatively short period of time. Perhaps much of the success achieved by the aviculturists at Slimbridge and elsewhere can be attributed in part to the practice of starting embryo development under domestic hens. We are currently testing this hypothesis with the 'radio egg' in the nest cup of broody hens.

It has been widely assumed in the past few years that water loss from incubating eggs is one of the most important factors in embryo survival and hatchability (Rahn & Paganelli 1981). Indeed Ar & Rahn (1980) concluded that 'water loss during incubation is mandatory if the relative content of an egg at the end of incubation is to remain essentially the same as at the beginning'. For water loss to occur there must be a gradient in the water vapour pressure across an eggshell. The many field records of approximately 16–18% loss of the initial mass of incubating eggs (Drent 1970) as well as studies with egg hygrometers (Rahn *et al.*, 1977) have confirmed the existence of such a gradient but not identified factors that influence it. The 'radio egg' showed that the steepness of the gradient was influenced by bird behaviour and nest materials. With the former, for example, it was assumed that the diffusion gradient across the shell of the Barnacle Goose egg was shallow between 1000–1830 h because the female came to the nest with wet plumage. In all the nests studied, bird behaviour leading to a high incidence of light admission to the nest cup caused the increase in the steepness of the diffusion gradient. Thus the 'radio egg' has identified bird behaviour as the principal cause of nest ventilation. The 'radio egg' also revealed that nest materials lost water throughout incubation and thereby accentuated the steepness of the water vapour pressure gradient across an eggshell. This is an important observation as far as aviculturists are concerned if the observations of Snyder & Birchard (1982) are confirmed. These workers have demonstrated that it is the extent of water loss during the first half of incubation of the eggs of domestic hens that determines hatchability. Their proposition 'that regulating the permeability of the eggshell may be important in preventing excessive drying of the inner shell membrane during early development' may need to be modified in the light of our findings.

The incubation strategies shown by the waterfowl included in this study were successful in that all, or almost all, the eggs hatched. Perhaps any advantages of single-parent incubation (with the unique addition of feather down as an insulation material), over the alternative condition of shared brooding, have to be sought in the

birds themselves rather than in their eggs. The bird's fitness to act as a heat source might be one variable that needs to be examined.

We wish to thank Sir Peter Scott C.B.E., D.S.C. for his continuing interest in the research discussed in this paper, Professor G. V. T. Matthews for his support over many years, the Science and Engineering Research Council for a grant and the many persons at the Wildfowl Trust who contributed. Finally we wish to thank Dr A. Robinson for advice on the methods used in the computer analyses.

REFERENCES

AR, A. & RAHN, H. 1980. Gas exchange of the avian egg: time structure and function. Amr. Zool. 20: 477–484.

BURKE, E. 1925. A study of incubation. Mont. Agric. Sta. Bull. 178: 1–44.

CAREY, C. 1980. The ecology of avian incubation. Bioscience 30: 819–824.

CHATFIELD 1975. The analysis of time series : theory and practice. Chapman & Hall . London.

CHATTOCK, A. P. 1925. On the physics of incubation. Phil. Trans. R. Soc. (London) Ser. B, 213: 397–450.

DRENT, R. H. 1970. Functional aspects of incubation in the herring gull. Behav. Suppl. 17: 1–132.

DRENT, R. H. 1973. The natural history of incubation. *In* Farner, D. S. (ed.,, Breeding biology of birds: 262–311. Washington, DC: National Academy of Sciences.

DRENT, R. H. 1975. Incubation. *In* Farner, D. S. & King, J. R. (eds), Avian biology, Vol. V: 333–420. New York: Academic Press.

HOWEY, P. W., BOARD, R. G. & KEAR, J. 1977. A pulse position modulated multichannel radio telemetry system for the study of the avian nest micro-climate. Biotelemetry 4: 169–180.

KEAR, J. 1970. The adaptive radiation of parental care in waterfowl. *In* Crook, J. (ed.), Social behaviour in birds and mammals: 357–392. London: Academic Press.

KEAR, J. 1972. Reproduction and family life. *In* Scott, P. & The Wildfowl Trust, The swans: 57–124. London: Michael Joseph.

KEAR, J. & MURTON, R. K. 1976. The origins of Australian waterfowl as indicated by their photoresponses. Proc. Int. Orn. Congr. 16: 83–97.

LANDAUER, W. 1967. The hatchability of chicken eggs as influenced by environment and heredity. Storrs Agric. St. Monogr., I (revised).

LUNDY, H. 1969. A review of the effects of temperature, humidity, turning and gaseous environment in the incubator on the hatchability of the hen's egg. *In* Carter, T. C. & Freeman, B. M. (eds), The fertility and hatchability of the hen's egg. Edinburgh: Oliver and Boyd.

RAHN, H., ACKERMAN, R. A. & PAGANELLI, C. V. 1977. Humidity in the avian nest and egg water loss during incubation. Physiol. Zool. 50: 269–283.

RAHN, H. & PAGANELLI, C. V. 1981. Gas exchange in avian eggs: collected works. Buffalo N. Y.: State University of New York at Buffalo.

ROBERTSON, I. S. 1961. The influence of turning on the hatchability of the hen's eggs. I. The effect of rate of turning on hatchability. J. Agric. Cambridge. 57: 49–56.

SCHWARTZ, A., WEAVER, J. D., SCOTT, N. R. & CADE, T. J. 1977. Measuring the temperature of eggs during incubation under captive falcons. J. Wildl. Mgmt. 41: 12–17.

SNYDER, G. K. & BIRCHARD, G. F. 1982. Water loss and survival in embryos of the domestic chicken. J. exp. Zool. 219: 115–117.

British Poultry Science, **22**: 407-408. 1981

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A NON-DESTRUCTIVE METHOD FOR STUDYING THE OUTER SURFACE OF AVIAN EGG SHELLS

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1. A technique is described whereby the surface of an egg shell can be examined repeatedly.

2. A dental impression compound is used to make a cast.

3. The cast can be examined directly or an impression made with nail varnish can be examined with the light or electron microscope.

INTRODUCTION

Casts have played a useful role in studies of the structure of egg shells since the beginning of the work by von Nathusius (see Tyler, 1964) who used Canada balsam. Casts prepared from collodion in acetone and stained with Mallory's triple stain were used by Tyler and Fowler (1978) in the studies which related egg-shell thickness to cone and core density. A plastic developed for the amateur modeller (Plasticraft, Turners, Leeds) was used by Board and Tullett (1975) to demonstrate the reticulate structure of the outer portion of the emu (*Dromaius novaehollandiae*) egg shell. Although these examples indicate the use of casts in studies of egg shells, their application is limited because the shell has to be dissolved to free the cast. A non-destructive method would allow repeated examination of the same shell and thus provide a means of studying the changes, if any, that might result for example from the washing or incubating of eggs. This communication outlines such a method.

MATERIALS AND METHODS

Xantopren plus, an organopolysiloxane material used for dental impressions (Bayer UK Ltd, Richmond, Surrey) and activator were mixed according to the manufacturer's instructions. The mixture was applied to the outer surface of an egg shell, allowed to harden and then removed. The cast was viewed with a dissecting microscope and overhead illumination. Alternatively an impression was made by painting the cast with "strong nail varnish with Nylon" (Chesebrough Pond's Ltd, London). The dry film of varnish was peeled off the cast and viewed with a microscope and transmitted light. It was also examined with a scanning electron microscope after being coated with gold.

The technique was used to study the surfaces of egg shells that had been examined previously (Board *et al.*, 1977; Board and Perrott, 1979).

OBSERVATIONS AND DISCUSSION

The general topological features of the surface of egg shells were seen during examination of a Xantopren cast with a dissecting microscope and overhead illumination. In general, however, a nail varnish impression of the cast was proved to be the most convenient method of studying shell structure. The impression, mounted on a microscope slide and overlaid with a coverslip, was examined with a standard light microscope. Indeed in terms of the gross features of the shell, there was little difference between photographs taken with the light or electron microscope (Plate-Fig. 1, A, B and C). The accuracy of reproduction of the method can be judged by comparing photographs taken of the cast of the outer surface of an egg shell and a piece of shell adjoining that which had been used to make an impression (Plate-Fig. 2, A and B). The vesicles in both photographs have a common size. No details of the light microscopes are given because equally good results were obtained with various models in our laboratory.

The technique discussed above offers a simple and accurate method for studying the surface structure of egg shells. Since it is non-destructive it can be applied in the repeated examination of egg shells that have been subjected to some treatment such as washing. Indeed the tough flexible properties of Xantopren allow casts to be made outside the laboratory with little fear of damage being caused by movement of the casts. In this context, the technique could well be of great importance in studies that seek to establish the extent and nature of changes occurring in the structure of the outer surface of eggs during incubation.

REFERENCES

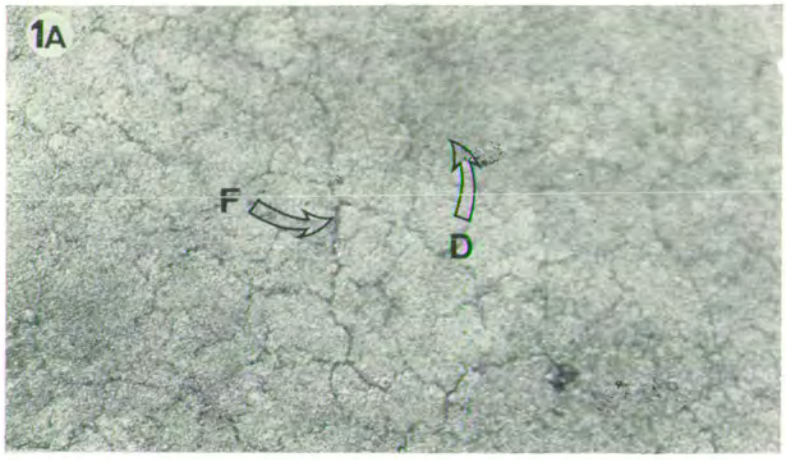
BOARD, R. G. AND PERROTT, H. R. (1979). Vaterite, a constituent of the eggshells of non parasitic cuckoos, *Guira guira* and *Crotophagi ani*. *Calcified Tissue International*, **29**: 63-69.

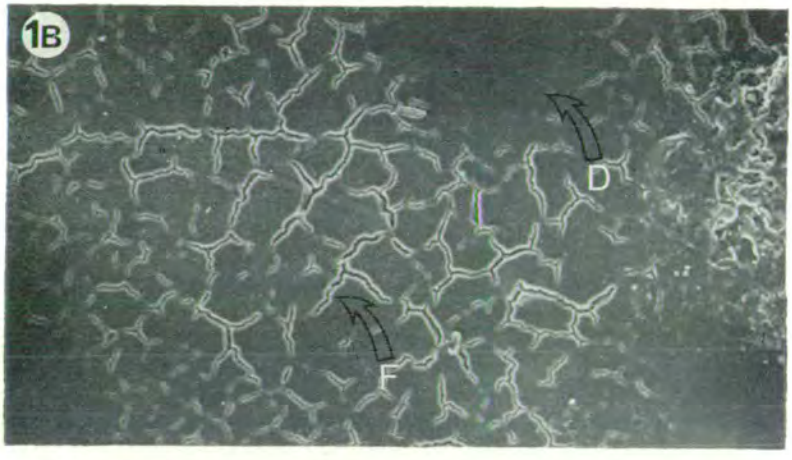
BOARD, R. G. AND TULLETT, S. G. (1975). The pore arrangement in the emu (*Dromaius novaehollandiae*) eggshell as shown by plastic models. *Journal of Microscopy*, **103**: 281-284.

BOARD, R. G., TULLETT, S. G. AND PERROTT, H. R. (1977). An arbitrary classification of the pore systems in avian eggshells. *Journal of Zoology, London*, **182**: 251-267.

TYLER, C. (1964). *Wilhelm von Nathusius 1821-1899 on Avian Eggshells. A Translated and Edited Version of His Work*. Reading, The University Press.

TYLER, C. AND FOWLER, S. (1978). The distribution of organic cores, cones, cone junctions and pores in the eggshells of wild birds. *Journal of Zoology, London*, **186**: 1-14.





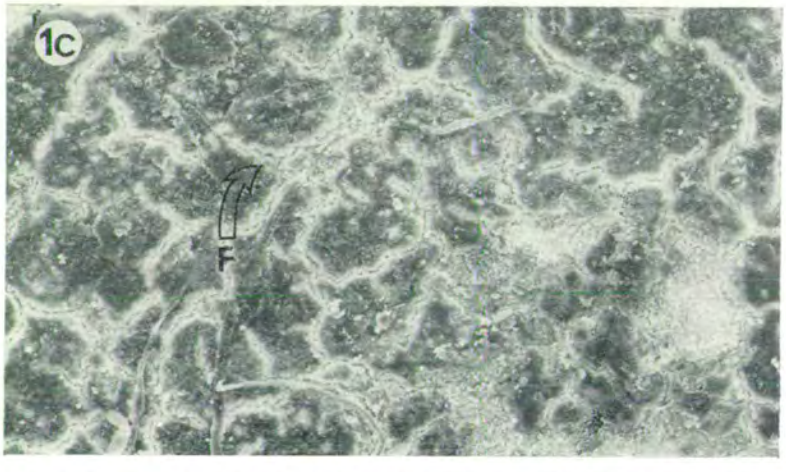


PLATE-FIG. 1.—The fine structure of the external surface of the egg shell of Pied-bill Grebe. The egg had been incubated naturally and was stained and highly polished. A. A nail varnish cast of a Xantopren cast of the Grebe egg shell examined with the light microscope (× 70). Fissures (F) and areas rendered featureless by dirt (D) are evident. B. A nail varnish cast of a Xantopren cast examined with scanning electron microscope (× 75). The fissures (F) and featureless areas (D) are evident. C. The external surface of the Grebe shell as seen with the scanning electron microscope (× 130). The fissures (F) are evident features.

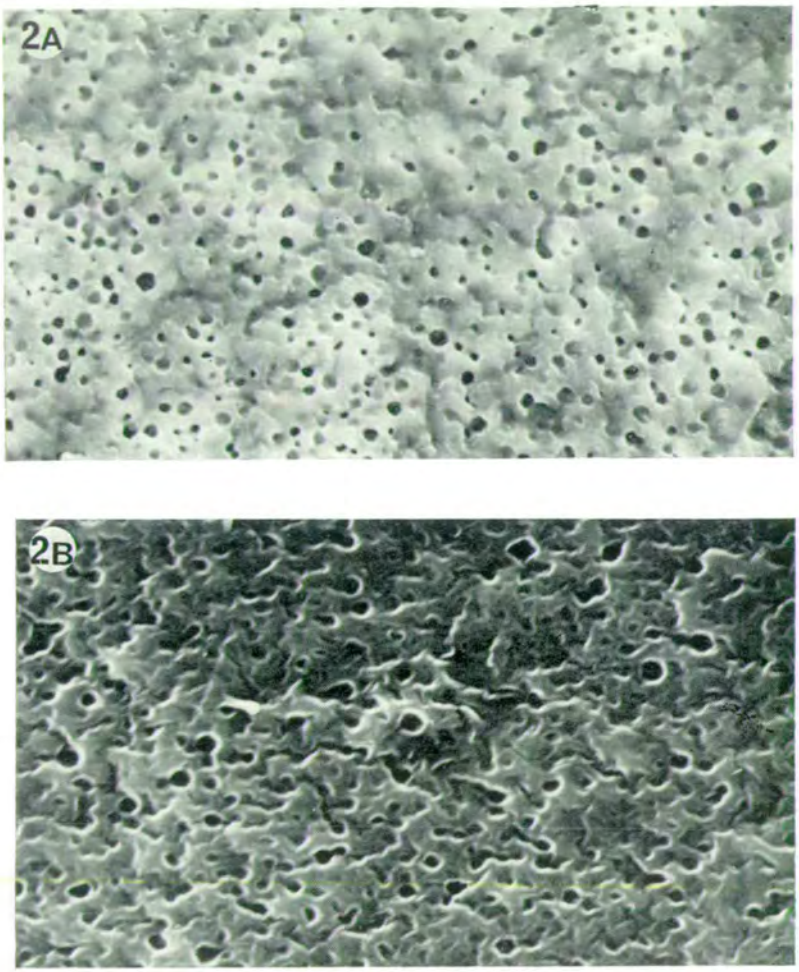


PLATE-FIG. 2.—The external surface of the shell of the egg of the passion bird. A. The surface as seen by direct examination of the shell with a scanning electron microscope (× 1600). B. A nail varnish cast of a Xantopren impression of an adjoining piece of shell (× 1600).

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The fine structure of the outer surface of the incubated eggshells of the Helmeted guinea fowl (*Numidia meleagris*)

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The surface of the eggshells of the Helmeted guinea fowl (*Numidia meleagris*) was polished during incubation by the parent. Examination with the light microscope showed that the cuticle had been removed from the ridges on the outer surface of the shell and that the plugs in the outer orifice of the pore canals had acquired extraneous materials including grease. Studies with a scanning electron microscope revealed that the spheres that made up the pore plugs retained their identity even though they were stained. It was concluded that ridges on the shell surface protected the pore plugs from damage by attrition and that the plugs acted as filters thereby preventing nest debris from occluding the pore canals or contaminating the shell membranes.

Contents

Introduction

Although the outer surface of the eggshells of many species of birds is covered with a stratum of spheres (Board, Tullett & Perrott, 1977; Board & Perrott, 1979*a*; Board *et al.*, in prep.), the adaptive significance, if any, of this layer has attracted little comment. Lack (1968) surmised that waterproofing would be an essential property of eggshells incubated in wet nests, such as those of grebes, or those that are wetted by water brought to the nest on the parents' plumage. He did not identify the reasons for this requirement nor did he define the mechanisms that waterproof eggshells, merely noting that eggs in wet nests have polished or powdery surfaces. The cuticle (a layer of glycoprotein spheres) on the outer surface of the eggshells of domestic hens prevents flooding of the pores when eggs are immersed in water at the same temperature (Board & Halls, 1973). The pores are flooded if work is done to overcome the resistance of the cuticle (Board, 1980). Flooding results, for example, when a partial vacuum is drawn and released suddenly in the head space of a closed vessel containing eggs immersed in water. As the filling of a few pores with contaminated water is the first

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stage in the infection of the contents of eggs (Board & Fuller, 1974), the cuticle can be considered to be a component of an egg's antimicrobial defence. If a large proportion of pores in an eggshell was flooded, then presumably the embryo would be asphyxiated.

Freedom from a requirement for an exogenous source of water is considered to have been the last major step in the evolution of the cleidoic egg of birds (Needham, 1931). As the eggshell needs to be porous so that the embryo can exchange respiratory gases with the atmosphere in the nest, it permits also the evaporation of water from the contents of the egg. Drent's (1975) review of field observations indicates that on average 16% of the water present at oviposition is lost by evaporation during incubation of an egg. The studies by Rahn and his collaborators (e.g. Ar & Rahn, 1978) have shown that this rather precise water loss in eggs of many species of birds results from an interplay of a shell's potential to conduct water vapour, egg mass, period of incubation, incubation temperature, barometric pressure and the relative humidity of the nest. The cuticle does not appear to influence the loss of water from the eggs of domestic hens (Board & Halls, 1973). If an eggshell containing many thousands of pores each having a cross sectional area of a few square microns only is considered in the context of the nest environment, then several substances that might be expected to block the pore canals and thereby diminish a shell's potential to conduct respiratory gases and water vapour can be tentatively identified: preening oils, mud, nest debris and dust arising from attrition between egg and egg, or egg and nest materials. Indeed should the blocking of pores be a cumulative process, then a shell's potential to conduct respiratory gases could be diminished to such an extent that the embryo's demands, which are maximal immediately before the inner membrane of the air cell is ruptured (Vleck, Vleck & Hoyt, 1980), might not be satisfied. It would seem reasonable therefore to consider the proposition that eggshells are adapted so that the pores are not blocked during incubation. This communication presents evidence that the layer of spheres on the surface of eggshells of the Helmeted guinea fowl (*Numidia meleagris*) serves such a purpose.

Materials and methods

Examination with the light microscope

Pieces of eggshell were examined with a Zenith MBS–1 Stereo microscope and overhead illumination. Waxoline green (1% w/v in chloroform; I.C.I., Manchester, U.K.) or Sudan black (0·3% w/v in 70% v/v ethanol; B.D.H. Poole, U.K.) were used to test for grease on the shell surface. The outer covers on the shells were removed by boiling in 5% (w/v) sodium hydroxide.

Examination with a scanning electron microscope

Pieces of shell, glued with silver paint (DAG 913, Acheson Colloids Ltd., Plymouth, U.K.) to stubs, were sputter-coated *in vacuo* with gold and examined on a JEOL (Japan) JSM 35C Scanning Electron Microscope. The accelerating voltages used in the examination of shells are given in the legends to the plates.

Results and observations

The eggs examined in this study were taken from the nest of a Helmeted guinea fowl (*Numidia meleagris*). Some eggs were taken before the onset of incubation; infertile and the shells of hatched eggs after the parents and keets left the nest—a scrape in the litter beneath

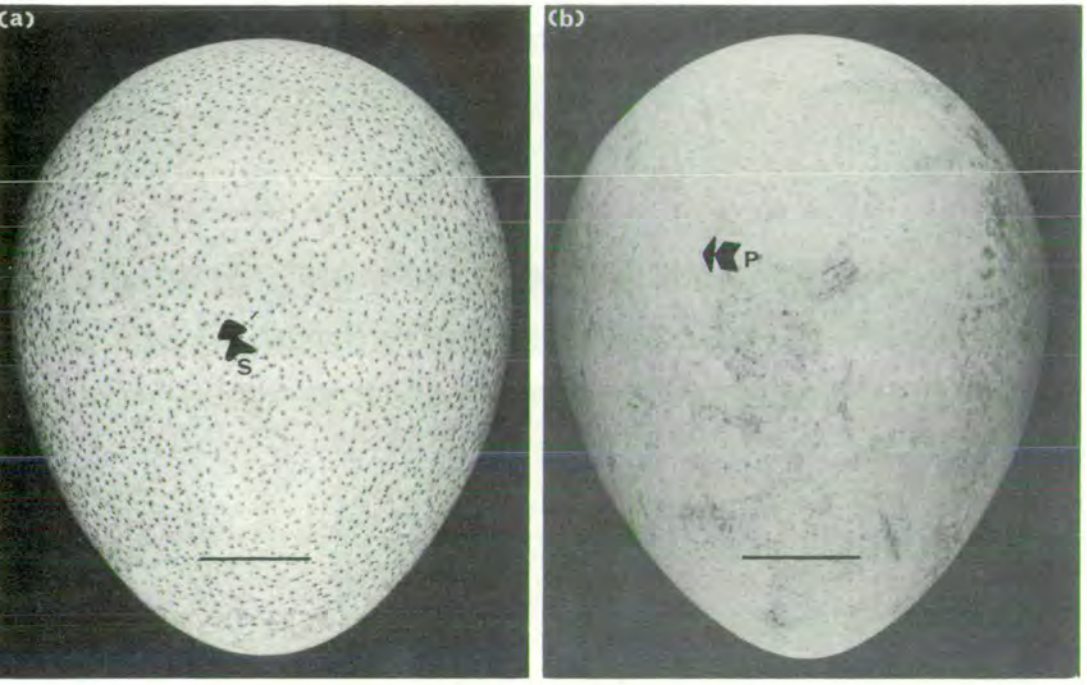

PLATE I. (a) Incubated egg of Helmeted guinea fowl (*Numidia meleagris*); the surface was polished and covered ith tan-coloured spots (s). (b) An unincubated egg showing mud smears and shallow pits (p). Bar marker, 1 cm.

ıe canopy of a small holly bush in the garden of one of the authors (R.G.B.). Plate I lustrates the gross changes which occurred in the appearance of the eggshells with ıcubation. The mud-smeared unincubated eggshells had a pitted surface and a matt finish hereas those of the incubated eggs were polished and covered with tan-coloured spots.

Observations with the light microscope

It was evident during examination with a light microscope and reflected light that there ere two types of brown spots on the incubated eggshells, small, irregularly shaped flecks, ıd larger round spots. Both were contained in depressions in the shell surface, the ridges round the depressions being scratched. As some of the shallow saucer-shaped depressions ıd a few of the external orifices of the pore canals were empty, it was assumed that the rown material had been scraped away, possibly when the eggs were turned. The pitted ppearance was the only notable feature of the shell of unincubated eggs. The brown spots on ıe shells of incubated eggs and the cover on unincubated ones were removed slowly by boil-ıg sodium hydroxide (5% w/v); the scoured shell surface was seen to have shallow epressions of irregular outline and relatively deep, saucer-shaped depressions at the xternal orifice of the pore canals.

The fat soluble dyes, Sudan black and Waxoline green, did not stain the surface of the shell f unincubated eggs, but they stained the fissures in the surfaces of the circular brown spots ı the shells of incubated ones. Soaking incubated shells overnight in petroleum ether

reduced the extent of staining of the fissures. When Waxoline green (1% w/v in chloroform was painted on the internal surface of an eggshell, it penetrated the pore canals and stained the outer surface of the shell. With unincubated eggs, a patch of material overlying a pore canal was stained deeply and it was evident from examination with the light microscope that there was lateral movement of the stain away from the heavily stained patch overlying a pore canal. The application of Waxoline green to the inner surface of the incubated shells did not cause gross staining of a shell's surface but stained fissures in the circular brown spots were seen with a light microscope.

Pore canals were evident in the radial faces of pieces of snapped shells of unincubated eggs but the resolution of the light microscope (used with reflected light) did not reveal the

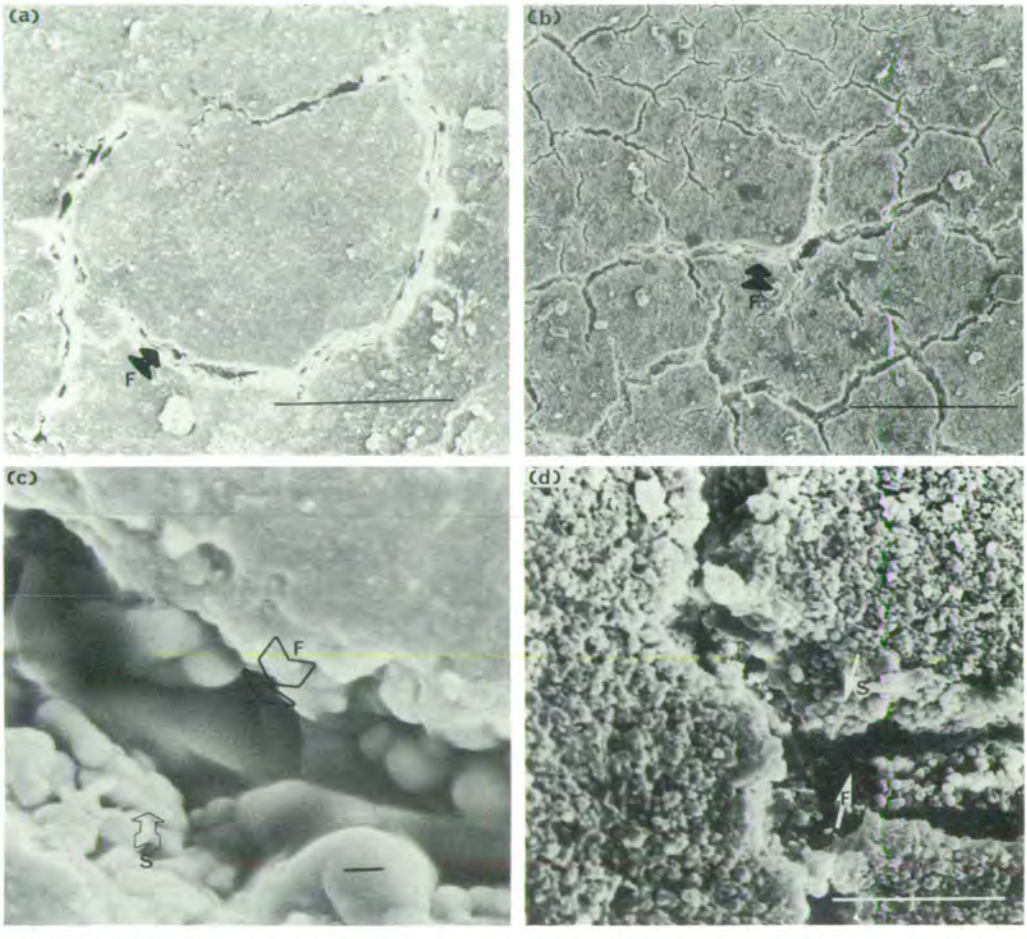

PLATE II. (a) The outer surface of the incubated eggshell of the Helmeted guinea fowl (*Numidia meleagris*); th surface was featureless apart from fissures (f) in the plug of cuticle in the outer pore orifice (bar marker, 100 μm accelerating voltage, 10 kV). (b) Unincubated eggshell with fissured outer surface (bar marker, 100 μm; acceleratin voltage, 10 kV). (c) Details of the faces of a fissure (f) and the outer cuticular on the surface of an incubated egg (ba marker, 1 μm; accelerating voltage, 10 kV). (d) Spheres (s) were a characteristic feature in the faces of th fissures (f) and on the surface of unincubated eggshells. Bar marker, 10 μm; accelerating voltage, 15 kV.

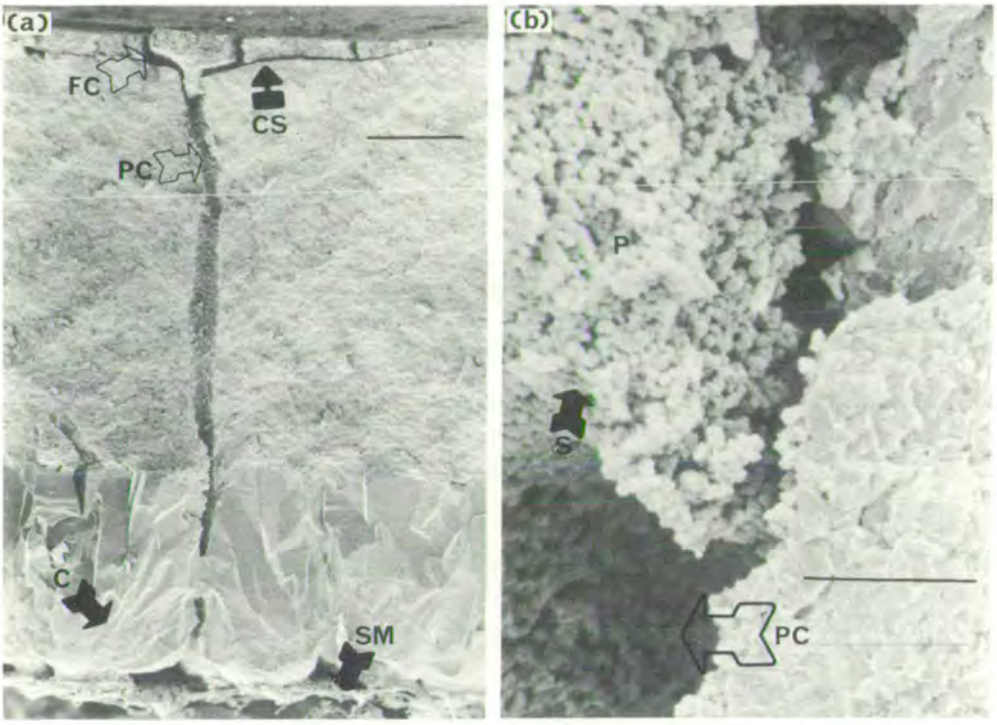

PLATE III. (a) Radial face of a snapped section of the shell of an unincubated egg of the Helmeted guinea fowl (*Numidia meleagris*) showing the counter-sunk area (cs) around the outer orifice of the pore canal (pc) which traversed the shell and terminated between cones (c) on the outer surface of the shell membranes (sm). Fissures (fc) in the cuticle were a notable feature (Bar marker, 100 μm; accelerating voltage, 10 kV). (b) Plug (p) of spheres (s) in the outer part of the pore canal (pc) of incubated eggshell. Bar marker, 10 μm; accelerating voltage, 10 kV.

detailed structure of the external pore orifice, apart from confirming that it was countersunk. With incubated eggs, a brown stain in the material extending down into the pore canal was a notable feature.

Observations with the electron microscope

The fine structure of incubated eggshells is shown in Plates II(a), (c) and of unincubated ones in Plates II(b), (d). As Plates II(a) and II(b) are of approximately the same magnification, direct comparison of the fine structure of incubated and unincubated shells is possible. It is evident from such a comparison that many of the fissures in the cuticle of incubated eggs have become filled with nest debris, preening oils, etc. Indeed the few remaining fissures were associated presumably with the cuticle overlying the outer orifice of the pore canals. It is evident from Plate II(d) that the cuticle on the guinea fowl eggshell is composed of spheres; with incubation the spaces between spheres are filled and even those in the radial face of a fissure are neither as discrete nor as numerous (Plate II(c)) as those in the walls of the fissures in the cuticle of unincubated eggshells (Plate II(d)). The gross appearance of the pore canals in the radial faces of snapped pieces of incubated or unincubated eggshells were similar, the shallow, counter-sunk external orifice filled with spheres being a notable feature in both

(Plate III(a)). It was evident with high powered magnification that plugs of spheres extende some way down into the pore canal, there always being a gap between the plug and the wa of the canal (Plate III(b)). Although the spaces between spheres and most of the fissures apa from those in the cuticle covering a pore were filled with debris during incubation (Plat II(a)), the spheres in the plug in the outer pore orifice retained their identity even though the had been stained brown, as was evident from examinations with the light microscope.

Discussion

This contribution is probably the first to present information on the changes occurring o the outer surface of eggshells during incubation. It was evident that this surface had bee scratched during incubation, possibly as a result of the eggs being turned. Not only ha attrition scratched the ridges on the calcitic shell but it had removed the cuticle from some o the shallow depressions and from a few of the pore orifices. In general, however, the oute orifice of the pore canal was plugged; presumably the plugs had been protected by thei location in the counter-sunk orifice. The loss of discrete fine structures, such as the sphere making up the cuticle, together with a reduction in the number of fissures in the cuticle provided evidence that the cuticle had been impregnated with nest debris and, judging fron the results obtained with fat stains, grease from the skin and feathers of the parent. Th observations made on shells, the inner surface of which had been painted with Waxolin green in chloroform, suggest that the area available for gaseous diffusion had bee diminished as a consequence of the deposition of nest debris. Thus the web of stained fissure in the pore plugs of unincubated eggshells contrasted markedly with the few fissures in thos of the incubated ones. Moreover the former stood out against a lightly stained backgroun whereas the latter appeared as lines on the tan coloured spots. This was taken as evidenc that debris had infiltrated the void spaces between the spheres on the surface of the incubate eggs. It was notable however that fissures were always present in the cuticle overlying th pore orifices of incubated eggs. The mechanism(s) responsible for preventing occlusion o these fissures have not been identified. The tan colour in the pore plugs of incubated egg indicated that debris had infiltrated through the entire plug without filling the void space between the spheres (Plate III(b)). Thus the plugs appeared to act as filters that preven extraneous material reaching the shell membranes. Although the guinea fowls nest—a scrape in the ground—would not appear to offer a particularly harsh environment to eggs the evidence discussed above does support the contention that their shells are adapted suc that attrition does not remove the pore plugs that act as filters thereby maintaining th diffusive capacity of the shell. These observations suggest that future studies of shel structure ought to consider incubated as well as unincubated eggs so that changes due t brooding are defined. Indeed the non-destructive technique for studying the fine structure o eggshells (Board, 1981) ought to allow observations to be made during incubation. Althoug information obtained by comparative studies of the type discussed above provides clue about the possible adaptive significance of the pore systems discussed by Board, Tullett & Perrott, (1977), Board & Perrott, (1979*a, b*), Board (1980) and Board, Perrott, Love & Scot (in prep.), a detailed picture will be built up only by combining field and laboratory obser vations. Indeed the possibility that shell adaptations may well contribute to the well-being o the embryo ought to be recognized by those who study the breeding biology of birds wher the emphasis has been placed largely on the behaviour of the parent(s) in relation to an egg'

need for turning, heat and protection (Drent, 1975; Board, in press; White & Kinney, 1974). A change in the emphasis in field observation could well identify features of bird behaviour that have evolved so that the conductivity of an adapted eggshell is not seriously impaired during incubation.

Studies with the light and electron microscopes showed that the cuticle of spheres on the surface of eggshells of the Helmeted guinea fowl (*Numidia meleagris*) were worn away from the ridges on the calcitic shell during incubation by the parents. The cuticle remaining in the depressions in the shell, especially the outer orifice of the pore canals, was coated on its outer surface with nest debris. Although the plug of spheres in the pore canals was stained, there was no evidence of the void spaces having been infiltrated with nest debris.

REFERENCES

Ar, A. & Rahn, H. (1978). Interdependence of gas conductance, incubation length and weight of avian egg. In *Respiratory function in birds, adult and embryonic*: 227–238. Piiper, J. (Ed.). Berlin: Springer.

Board, R. G. (1980). The avian eggshell—a resistance network. *J. appl. Bact.* **48:** 303–313.

Board, R. G. (1981). A non-destructive method for studying the outer surface of avian eggshells. *Br. Poult. Sci.* **22:** 407–408.

Board, R. G. (In press). Properties of avian eggshells and their adaptive value. *Biol. Rev.* **57.**

Board. R. G. & Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biol. Rev.* **49:** 15–49.

Board, R. G. & Halls, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *Br. Poult. Sci.* **14:** 69–97.

Board, R. G. & Perrott, H. R. (1979*a*). The plugged pores of tinamou (Tinamidae) and Jaçana (Jananidae) eggshells. *Ibis* **121:** 469–474.

Board, R. G. & Perrott, H. R. (1979*b*). Vaterite, a constituent of the eggshells of the non-parasitic Cuckoos, *Guira guira* and *Crotophagi ani. Calc. Tissue Int.* **29:** 63–69.

Board, R. G., Perrott, H. R., Love, G. & Scott, V. D. (In prep.). *The phosphate rich cover of the eggshells of the Great crested grebe,* ***Podiceps cristatus,*** *and related species.*

Board, R. G., Tullett, S. G. & Perrott, H. R. (1977). An arbitrary classification of the pore system in avian eggshells. *J. Zool., Lond.* **182:** 251–265.

Drent, H. R. (1975). Incubation. In *Avian biology* **5:** 333–420. Farner, D. S. and King, J. R. (Eds). New York: Academic Press.

Lack, D. (1968). *Ecological adaptations for breeding birds.* London: Chapman and Hall.

Needham, J. (1931). *Chemical embryology.* 3 vols. Cambridge: University Press.

Vleck, C. M., Vleck, D. & Hoyt, D. F. (1980). Patterns of metabolism and growth in avian embryos. *Am. Zool.* **20:** 405–416.

White, F. N. & Kinney, J. L. (1974). Avian incubation. *Science, N. Y.* **186:** 107–115.

SECTION 5

MICROBIOLOGY

Discussions of the evolution of cleidoic eggs such as those of birds tend to lay stress on such eggs achieving independence of a need for exogenous supplies of water. The fact that such eggs commonly contain relatively large food reserves and may thus be vulnerable to microbial colonization rarely if ever attracts a comment in the zoological literature. The off-prints contained in this section deal with the phenotypic properties of the micro-organisms that cause the addling of eggs and discuss in detail the form of the antimicrobial defence systems which protect the yolk, the major food reserve, from infection.

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NON-SPECIFIC ANTIMICROBIAL DEFENCES OF THE AVIAN EGG, EMBRYO AND NEONATE

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CONTENTS

I. INTRODUCTION

Unlike viviparous animals, birds are dependent for hatching success upon the development of the embryo in an environment isolated from that of the parents by the eggshell and associated membranes, there being nothing akin to the placenta of mammals. In addition to the nutrients required for prenatal development and the maintenance of the chick for a few hours or days after hatching, the egg contains a large amount of water. During the brooding period, the cleidoic egg of the majority of birds needs parental attentiveness (egg turning and heat), an extrinsic source of oxygen and, perhaps, a suitable relative humidity (RH). The requirement for a large and nutritionally complete food reserve in the eggs imposes a heavy demand on the female's own nutrient reserves as well as on her time during the laying of a clutch. The magnitude of

this demand has long been recognized and Lack (1968) has discussed the ecological adaptations associated with it. Likewise the vulnerability of the egg, and indeed of the parents also, to predation has been appreciated. Markings on the shell have been interpreted as camouflage, and the location of nesting sites and the behaviour of the parents at times of danger as defence. It is suggested that undue emphasis has been given to 'macro-predators'. In some surveys (e.g. that of Drent (1967) on the herring gull) the numbers of addled eggs were of the same magnitude as those lost by predation. From a purely theoretical viewpoint, there would appear to be a need for the food reserves of the cleidoic egg to be shielded from exploitation by micro-organisms. Moreover, a similar requirement would seem to exist for the embryo and neonate until such times as its own cellular or humoral systems can provide an effective defence against microbial infection.

It has been known for many years that the chick embryo increases in resistance to infection as it develops. For example, Western equine encephalomyelitis (Bang, 1943) and lymphocytic choriomeningitis (Volkert & Larson, 1965) which are lethal for 7–11-day embryos, are innocuous when injected into those of 15–17 days. Solomon (1971) lists many more such examples. In spite of this increasing resistance of the embryo to infection it is difficult to demonstrate immunocompetence experimentally until about the time the chicken hatches (van Alten & Schechtman, 1963; Solomon, 1964, 1966). After hatching the chick also goes through a stage where it becomes less susceptible to certain infections. Thus micro-organisms such as the pneumococcus (Andrews & McKinnon, 1961), *Salmonella gallinarum* (Solomon, 1968*a*), *Streptococcus faecalis* (Fuller & Jayne-Williams, 1970*a*) and Aujeszky's virus (Ramachandran & Fraser, 1971) are all more virulent for newly hatched chicks than for week-old ones.

In some instances these changes in resistance of the embryo and neonate cannot be adequately explained in terms of an immunological response and it is suggested that non-specific mechanisms are operative. This is particularly the case with the embryo but may well also apply to the neonate which has yet to reach full immunological maturity. The following review beings together information on the non-specific antimicrobial defences of the egg, embryo and neonatal bird, which we feel may present an alternative explanation for the changes in resistance associated with embryogenesis and age of the neonatal bird.

II. THE EGG

In the absence of an adequate immune response and the localized nature of the protection afforded by the maternal antibody (Yamomoto & Bigland, 1966), the developing embryo must rely heavily on non-specific defence mechanisms. The following section discusses those antimicrobial defence systems which do not depend on the specific recognition of antigen. The information is drawn largely from studies of infertile hen's eggs, but much of it will apply equally well to the fertile egg. Comparative studies of shells (Tyler, 1969*b*) and of albumen (Sibley, 1960, 1970) of eggs of other species suggest that the principles derived from poultry research may well have general application. The majority of eggs of domestic hens contain no viable organisms at oviposition (Brooks & Taylor, 1955) and they are likely to harbour few organisms even

when congenital contamination has occurred (Harry, 1963*a*, *b*). Under commercial conditions of intensive incubation and rearing, pathogens transmitted through the egg (Payne, 1968) can infect chicks (Landauer, 1967). Although such transmission may well occur in nature, it would presumably be important only in birds nesting in flocks where there would be opportunities for the onset of epidemics. Is it in this situation that maternal antibodies provide passive immunity to chicks being reared by parents who are carrying specific pathogens?

After separation from the mother at oviposition, the egg is exposed constantly to contamination with bacteria, viruses and moulds over which the parents have little or no control unless preening oils play a role. There appears to be a high specificity (organotropism) between the type of bacterium and the type of cells infected in the embryo (Goodpasture & Anderson, 1937). The extra-embryonic membranes by enclosing the embryo may play an important role in protecting it against bacteria which lack the ability to colonize and penetrate tissues. As the natural period of egg laying and incubation occupies only a small part of any year, there would appear to be little opportunity for the selection, even in areas used by flocks, of bacteria or moulds which have invasive capacity. Moreover, as the first embryonic membrane, the blastoderm, is not complete until the second or third day of incubation of the hen's egg (New, 1959), the wellbeing of the embryo, and particularly of those eggs laid at the beginning of a clutch, must be dependent upon some other form of antimicrobial defence. Even when the yolk is enclosed by the blastoderm, it is surrounded by a solution of proteins, carbohydrates and trace metals which might be expected to provide a medium for microbial growth. Such growth could be harmful to the embryo both immediately through lowering the redox of the albumen at a time when the embryo is about to enter the first period of respiratory crisis and subsequently when the albumen moves *via* the sero-amniotic connexion to the amnion, lungs and air sacs (Witschi, 1956). Thus any antimicrobial components of the albumen would have to be non-toxic to embryonic tissues.

There is a requirement for the embryo in the eggs of all oviparous animals to develop in a germ-free environment. Although this appears to have been a neglected field of study, the available information suggests that this end is achieved in several ways. With the eggs of salmon (*Oncorhynchus*), the radiate membrane or 'capsule' is colonized by a characteristic bacterial flora and the wellbeing of the embryo is presumed to depend upon a dynamic ecosystem (oosphere) precluding colonization of the egg by pathogens (Bell, Hoskins & Hodgkiss, 1971). It is notable that the microflora of the effluent water from hatcheries is dominated by members of the oosphere (Trust, 1972). In the albumen of fish and snail eggs there are substances (protectins) which through being antimicrobial may well have a role in the defence of the embryo (Uhlenbruck, Springer, Herman & Franke, 1972) and it has been shown that the jelly surrounding the eggs of amphibia has antimicrobial properties (Gabayeva, 1962).

In a review of microbial infection of the hen's egg, it was suggested (Board, 1969) that the yolk in a cleidoic egg such as that of a bird is shielded from microbial colonization through the combined workings of physical (the shell and shell membranes) and chemical (the albumen) systems. These have been selected presumably so that,

together with the micro-environment created by the parents' nesting habits (Drent, 1972), breeding success is dependent upon a high hatchability of the few eggs per pair per season – birds, unlike most fishes, reptiles and amphibia, have been freed from the statistical demands implicit in the parable of the sower. The evolution of a defence system must have occurred alongside those selective processes which favoured the physiological demands of the embryo. On occasions, as with the shell (see below), conflict between two or more demands has resulted apparently in a compromise. In this section, an attempt has been made to study the relation between the defence systems and some of the major physiological and physical demands of the embryo.

(1) *The shell*

Some of the many demands which the avian embryo makes on the individual components of the egg are listed in Table 1. An example of conflict in these demands is provided by the shell where optimal exchange of respiratory gases, for example, calls for adequate porosity without a concomitant weakening of the shell. It has been suggested (Tyler, 1955) that this may account for the distribution of pores in the shell of the hen's egg – "the distribution...in almost every case is non-random and tends towards uniformity and away from aggregation" (Tyler, 1969*a*). Even when malformation does not result in the fragility of eggs, the hatchability is reduced in those having 'porous' shells (Rauch, 1952; Landauer, 1967). The term porosity has acquired a nebulous meaning, as pointed out by Board & Halls (1973*a*), and it is not clear from these reports whether the number of pores per shell, or the total *area* of pores per shell was responsible for the embryo failing to develop. From a microbiological point of view, the integrity of the shell is obviously important; the contents of cracked eggs are easily contaminated (Vadehra, Baker & Naylor, 1969) and Drent (1967) observed a high incidence of cracked shells among the addled eggs on the breeding grounds of the herring gull, *Larus argentatus* Pont.

Gaseous exchange must presumably be accommodated without the shell being rendered liable either to waterlogging or to excessive loss of water by evaporation. Board & Halls (1973*a*, *b*) found that the cuticle (bloom) plays an important role in impeding the movement of water across the shells of the eggs of the domestic hen, mallard, quail and guinea-fowl. It did not control the rate of evaporation. They noted, however, that all the eggs included in the survey had a few pores (1–40) which were flooded when eggs were subjected to a hydrostatic pressure of 0·35 kg/cm^2, and that those lacking cuticle sometimes had hundreds of pores flooded. These authors surmised that the cuticle functioned in part by capping the outer surface of the pores and in part by plugging the pore canals. In subsequent studies (Board, unpublished) it has been shown that the eggs of certain commercial strains of laying hens have a well-formed cuticle, as shown by the staining reaction of the whole shell, but a low resistance to water under the pressure noted above. Until such time as there are reliable techniques for demonstrating the presence of cuticle in thin radial sections of shells, it seems reasonable to assume that the shell is rendered water-resistant by material (cuticle ?) lodged in the pore canals. Support for such an assumption is given

Table 1. *The roles of components of the egg in the wellbeing and development of the embryo*

Component	Physiological function	Contribution to: Physical protection	Contribution to: Antimicrobial defence
1. Cuticle, shell and shell membranes	(*a*) Exchange of respiratory gases	(*a*) Protection against crushing	(*a*) Barrier to microbial invasion
	(*b*) Conservation of water	(*b*) Prevention of waterlogging	
	(*c*) Reservoir of calcium	(*c*) Camouflage	
	(*d*) Insulation?		
2. Air cell	(*a*) Air reservoir	(*a*) Compensation for changes in pressure	—
3. Thin white and albuminous sac	(*a*) Reservoir of water	(*a*) Cushioning against damage due to sudden and violent movement	(*a*) Holding yolk in central position
	(*b*) Gaseous exchange with the embryo	(*b*) Lag against violent fluctuations in temperature	(*b*) Viscosity and impediment to bacterial movement
	(*c*) Source of protein		(*c*) Control of rate and extent of microbial growth
			(*d*) Passive immunity of chick
4. Chalazae and chalaziferous membrane	—	—	(*a*) Holding yolk in central position
5. Vitelline membrane	—	—	(*a*) Physical isolation of yolk from white
6. Yolk	(*a*) Principal depot of all major and micro-nutrients	(*a*) Location of young embryo at least distance from heat	(*a*) Passive immunity of chick

by Vadehra, Baker & Naylor (1970*a*). They noted a higher incidence of rotting in eggs taken *post mortem* from the uterus than in those from which the cuticle had been removed with ethylenediamine tetra-acetic acid (EDTA). Cuticle at the surface of the shell might play a part in water-repellency and it is probable that the efficiency of such a function is increased by preening oil derived from the parent's feathers. Thus one of us (R. G. Board) has noted that pheasant eggs taken from a brooding bantam are more water-repellent than those taken immediately after they were laid. Further evidence that there is probably a need to differentiate between water-resistance and water-repellency of the shell of avian eggs has come from studies of the eggs of pigeon (R. G. Board, unpublished observations). Such eggs do not have cuticle and open pores are easily seen with the scanning electron microscope. The shell of the pigeon egg is water-repellent in the sense that a drop of coloured water will flow from its surface without penetrating the pores. It requires only a slight hydrostatic pressure to force water through the pores (the actual pressure required has not been determined because of the difficulty in obtaining sufficient eggs for experimental purposes).

Moreover, it has been shown that drops of water falling from a height of about 2 metres overcome the apparently negligible water-resistance of the shell of pigeon eggs. These observations suggest that climatic conditions and the nesting site of birds may have played important roles in the evolution of the eggs of different species of birds. Thus the shells of the eggs of ground-nesting species such as hens and pheasants have the properties of water-resistance and water-repellency whereas those of a tree-nesting species such as the pigeon have water-repellency only.

It has been shown that liquid uptake by the eggs of domestic hens is greater in areas where the shell is rough or knobbly (Alls, Benton, Krauss & Cover, 1963; Alls, Cover, Benton & Krauss, 1964). Pores which are flooded when eggs are immersed in water also permit the invasion of minute particles such as carbon black and bacteria (Board & Halls, 1973*a*). Faults in the capping and/or plugging of a few pores (about 40 out of $7–17 \times 10^3$ in the eggs of domestic hens having both water-resistance and water-repellency) render shells liable to fatal bacterial infections, and the opportunity for penetration is increased greatly by malformation of the shell. In the absence of cuticle, the egg appears to be particularly susceptible to infection.

(2) *The shell membranes*

The shell membranes, consisting as they do of layers of anastomosing fibres of keratin encapsulated in a glycoprotein (Cooke & Balch, 1970), may be considered as a bundle of capillaries having pores of about 1 μm diameter (Wolken, 1951; Bellairs & Boyd, 1969; Fujii & Tamura, 1970; Tung & Richards, 1972) with properties akin to a semi-permeable membrane. The establishment of a thread of water along a pore canal might be expected to lead to its continuous movement (and the translocation of bacteria) because of osmotic forces acting across the shell membranes. It has not been possible to demonstrate such a phenomenon in whole eggs, presumably because the drag imposed by the pore canal is greater than the osmotic forces, but extensive uptake occurs in cracked eggs (Board & Halls, 1973*a*). This may well account in part for the high levels of contamination of the contents of such eggs (Vadehra *et al.*, 1969).

If the contents of the eggs of domestic hens are replaced with an aqueous suspension of bacteria and a negative pressure is applied to the shell, micro-organisms are not present in the filtrate unless the shell membranes have been removed (Haines & Moran, 1940; Garibaldi, 1960). These findings have led to the view that the shell membranes behave as bacterial filters, but it has to be emphasized that in such experiments the membranes are compressed and possibly forced into the pore canals. In experiments where pressure has not been applied (Williams & Whittemore, 1967; Williams, Dillard & Hall, 1968), the membranes impede the movement of bacteria only for a limited period. When whole eggs were challenged externally with large numbers of bacteria, the organisms were recovered from the inner surface of the inner shell membrane within minutes of the challenge (Bean & MacLaury, 1959). These observations suggest that the shell membranes are capable of imposing only a temporary barrier to the inward movement of bacteria. The membranes probably offer no barrier at all to the infiltrating hyphae of moulds. Once bacteria have passed through the

shell membranes, the viscosity of the albumen ensures that they remain in a clump (Gillespie & Scott, 1950) and, in fresh eggs, the combined workings of the chalazae and albuminous sac preclude contact of the contaminants with the yolk.

(3) *Albumen*

This discussion of the functions of the shell and its membranes indicates that the conflicting demands for shell strength, allied with adequate porosity for the exchange of respiratory gases without excessive evaporation or waterlogging, have resulted in an evolutionary compromise and an integument across which micro-organisms can be translocated. Thus contamination of the albumen is likely to be increased by any agent which promotes translocation (see below). Moreover, the yolk remains at risk until it is enclosed in the extra-embryonic membranes.

(*a*) *Albumen as growth medium*

Early discussions of the inadequacy of the white of the hen's egg as a medium for microbial growth emphasized its alkaline (pH 9–10) reaction (Sharp & Whitaker, 1927) and its low content of non-protein nitrogen (Haines, 1939). The albumen of the fresh eggs of domestic hens contains about 0·14–0·54 μmoles amino acids/ml and, after storage for several weeks, up to 2·3 μmoles/ml (Ducay, Kline & Mandeles, 1960). The origin of the amino acids contributing to the increase with storage has not been determined, but these authors inferred that they may have diffused out from the yolk. The yolk is isolated from the albumen by the vitelline membrane. This consists of proteins arranged as a three-dimensional network in an inner layer and a lattice work of fine fibrils in an outer layer (Bellairs, Harkness & Harkness, 1963). The isolated vitelline membrane acts as a semi-permeable membrane, but the osmotic pressure gradient between yolk and albumen is steep (Moran & Hale, 1936). In conjunction with the diffusion gradient in the yolk (Smith, 1934), the membrane contributes to the limitation of exchange of materials between the yolk and white. When this barrier is destroyed, as happens in eggs laid by hens feeding on cycloprenoid compounds such as sterculic acid (Phelps, Shenstone, Kemmerer & Evans, 1965), the outward flow of Fe^{3+} causes the albumen to be deeply stained by the chromogen formed by the Fe^{3+}–ovotransferrin complex, and the inward flow of water causes a distension of the yolk.

It has been established that an abrupt change in the temperature (cold shock) or pH (alkaline shock) of a suspending medium causes a range of chemicals to be released from bacteria. The addition of bacteria to normal (pH 9·5) albumen (Board & Halls, 1973*c*) causes the release of ninhydrin-positive substances. This may well increase the micro-organisms' requirement for the simple nitrogenous substances necessary for the initiation of growth. Moreover, if shocking released biotin and riboflavin, the organism would be unable to reclaim these because of their union with avidin and 'apoprotein' respectively. There is perhaps a danger in assuming that the anti-proteolytic factors in the albumen (Table 2) will prevent micro-organisms from releasing simple nitrogenous compounds from the albumen. The alternative and perhaps more acceptable

Table 2. *The albumen considered as a medium for microbial growth*

A. *Content of nutrients**

(1) Gross composition (%): water, 88·1; protein, 10·2; fat, 0·05; ash, 0·6.

(2) Major proteins (% of total): ovalbumin, 63·8; conalbumin, 13·7; ovomucoid, 10·1; lysozyme, 3·5; ovoglobulin, 7·5; ovomucin, 1·4; avidin, 0·1.

(3) Free sugars (almost entirely glucose); 0·4 % (w/v).

(4) Growth factors: riboflavin, 3·6 (μg/g); nicotinic acid, 0·93 (μg/g); pantothenic acid, 3·0 (μg/g); biotin, 0·07 (μg/g); vitamin B_{12}, 0·75 (mμg/g); folic acid, 0·015 (μg/g); vitamin B_6, 2·2 (μg/g).

(5) Free amino acids**: 0·14–0·54 μmoles/ml.

B. *Bacteriostatic and bactericidal substances*

Lysozyme: hydrolyses the peptidoglycan of the cell wall of prokaryotes.
Avidin: combines with biotin, thereby making it unavailable to micro-organisms.
Apoprotein: combines with riboflavin, thereby making it unavailable to micro-organisms.
Ovotransferrin: chelates Fe^{+++}, Cu^{++} and Zn^{++}, pH 9·6: causes alkaline shock to bacterial cells.

C. *Other components****

Ovoinhibitor: inhibits fungal proteases.
Ovomucoid: inhibits trypsin.
Component A: inhibits trypsin and chymotrypsin.
Component B: combines with vitamin B_6.
Component C: chelates Ca^{++}, Mg^{++}.
Component D: inhibits ficin and papain.

* Mean values taken from Long (1968). ** Mean values taken from Ducay *et al.* (1960). *** Some of these have been considered to be components of the antimicrobial defence, but there is no direct evidence to support such claims.

interpretation is that they prevent degradation by enzymes released from autolysing spermatozoa or from cells in the amniotic membrane.

(*b*) *Conalbumin*

The observation that the albumen of the hen's egg contains an iron-binding agent (Alderton, Ward & Fevold, 1946), conalbumin (ovotransferrin) (Schade & Caroline, 1944), provided the first substantial evidence to account for the failure of bacteria to multiply in the albumen. Ovotransferrins have been observed in the albumen of eggs of all birds so far examined (e.g. Sibley, 1960, 1970). With albumen *in vitro*, extensive bacterial growth does not occur unless ovotransferrin is quenched with Fe^{3+} (Garibaldi, 1960) or its action negated by iron-transport compounds (Garibaldi, 1970). Growth of the test organisms in albumen equalling that in a nutrient broth is not achieved unless the Fe^{3+}-supplemented albumen contains additional nitrogen compounds, yeast extract (Seviour & Board, 1972) or ammonium ions (Board & Halls, 1973*c*). With hen's eggs held at room temperature, bacterial growth in the albumen is promoted by the injection of iron into the albumen (Board, 1964).

Studies of micro-organisms grown in a nutrient medium containing ovotransferrin (Theodore & Schade, 1965*b*) indicate that the consequent iron-deprivation causes a switch from respiration to glycolysis in a facultative anaerobic bacterium such as *Staphylococcus aureus*. This is associated with an extended lag phase of growth and an exponential phase of limited duration after growth starts (Theodore & Schade, 1965*a*).

A small inoculum does not initiate growth in normal albumen (Board & Halls,

1973*c*), H-ions do not accumulate and the glucose is not used (Seviour & Board, 1972). In these experiments the organisms were disseminated in the albumen, whereas in an infected egg they would presumably be maintained in a clump (Gillespie & Scott, 1950) because of the viscosity of the white. When measuring the H-ion or O_2 concentration of albumen, we have noted that the viscosity causes a steep diffusion gradient to be formed rapidly around an electrode. Thus a clump of facultatively anaerobic bacteria in albumen might be expected to produce a zone of low redox potential, thereby accentuating a tendency to switch from respiration to glycolysis. It is noteworthy that the albumen of the eggs of domestic hens, mallards, pintails, gadwalls and Canada geese (Seviour & Board, 1972) impedes the fermentation of glucose by a heavy suspension of lysozyme-resistant bacteria. This can be overcome by supplementing the albumen with yeast extract, but Fe^{3+} has little effect in this situation. It has been noted (Board & Halls, 1973*c*) that ammonium ions enhance the fermentation of the glucose in albumen seeded with large numbers of *Escherichia coli*, but this can be prevented by the addition of an inhibitor of protein synthesis such as chloramphenicol. These observations suggest that the growth of bacteria in fresh albumen is hindered by an interplay of pH (both *per se* and through its enhancement of the chelating potential of ovotransferrin), iron-deprivation and lack of adequate amounts of non-protein nitrogenous compounds.

(*c*) *Lysozyme*

Laschtschenko (1909) observed rapid lysis of *Bacillus* spp. added to the albumen of the hen's egg. The enzymic nature of the lysis was recognized by Fleming (1922) and he proposed the name lysozyme for the lytic agent. This class of enzyme is present in the albumen of the majority of birds' eggs (e.g. Sibley, 1960, 1970) but there are differences in amino-acid sequences and immunological properties of the enzymes obtained from different species (e.g. Prager, Arnheim, Mross & Wilson, 1972). Moreover, the concentration of lysozyme, as determined by gel-diffusion methods, varies with species (Fleming & Allinson, 1924; Smolelis & Hartsell, 1951). Lysozyme hydrolyses the β1–4 linkage of the polymers formed by alternating units of N-acetyl muramic acid and glucosamine in the peptidoglycan of the cell envelopes of prokaryotes (Rogers & Perkins, 1968). This heteropolymer forms a rigid network whereby the cell is given, among other things, shape and protection against osmotic lysis. Although the lytic action of lysozyme in albumen (Fleming, 1922; Garibaldi, 1960) has been demonstrated with lysozyme-sensitive bacteria, there is no direct evidence that it plays an important role in protecting avian eggs against infection. In fact the Russian workers Girfanova (1949*a*, *b*), Korotkova (1957*a*), Tokin (1964) and Mouchan (1964), have emphasized time and again that this enzyme has but a minor role in the egg's antimicrobial defence. Moreover, there is no evidence (Board & Halls, 1973*c*) that the alkaline albumen renders lysozyme-resistant organisms sensitive, as happens when they are suspended in tris buffer (pH 8·0) containing ethylenediamine tetra-acetic acid and lysozyme (see Vos, 1964).

It is possible that lysozyme may be involved in the physical rather than in the chemical defence of the egg against infection. Brooks & Hale (1959, 1961) were unable

to associate thinning of the thick white with depolymerization of ovomucin, a glycoprotein which forms a network throughout the albuminous sac. They were of the opinion that the viscosity of the white resulted from a network formed from ovomucin and lysozyme, an interaction which had been suggested by Hawthorne (1950) and Cotterill & Winter (1955). Soluble derivatives of ovomucin can interact with stoichiometric amounts of lysozyme through salt-bridges (Robinson & Monsey, 1969*a*, *b*). Moreover it has been shown that there are several components in the glycoprotein, one of which, β-ovomucin, is converted to a soluble form during the thinning of egg white stored at 37 °C (Robinson & Monsey, 1971, 1972). Perhaps this is the means whereby the thick white has its viscosity lowered in readiness for transfer through the sero-amniotic connexion to the amnion. Before this event, the albuminous sac must be a barrier to the movement of micro-organisms within the egg.

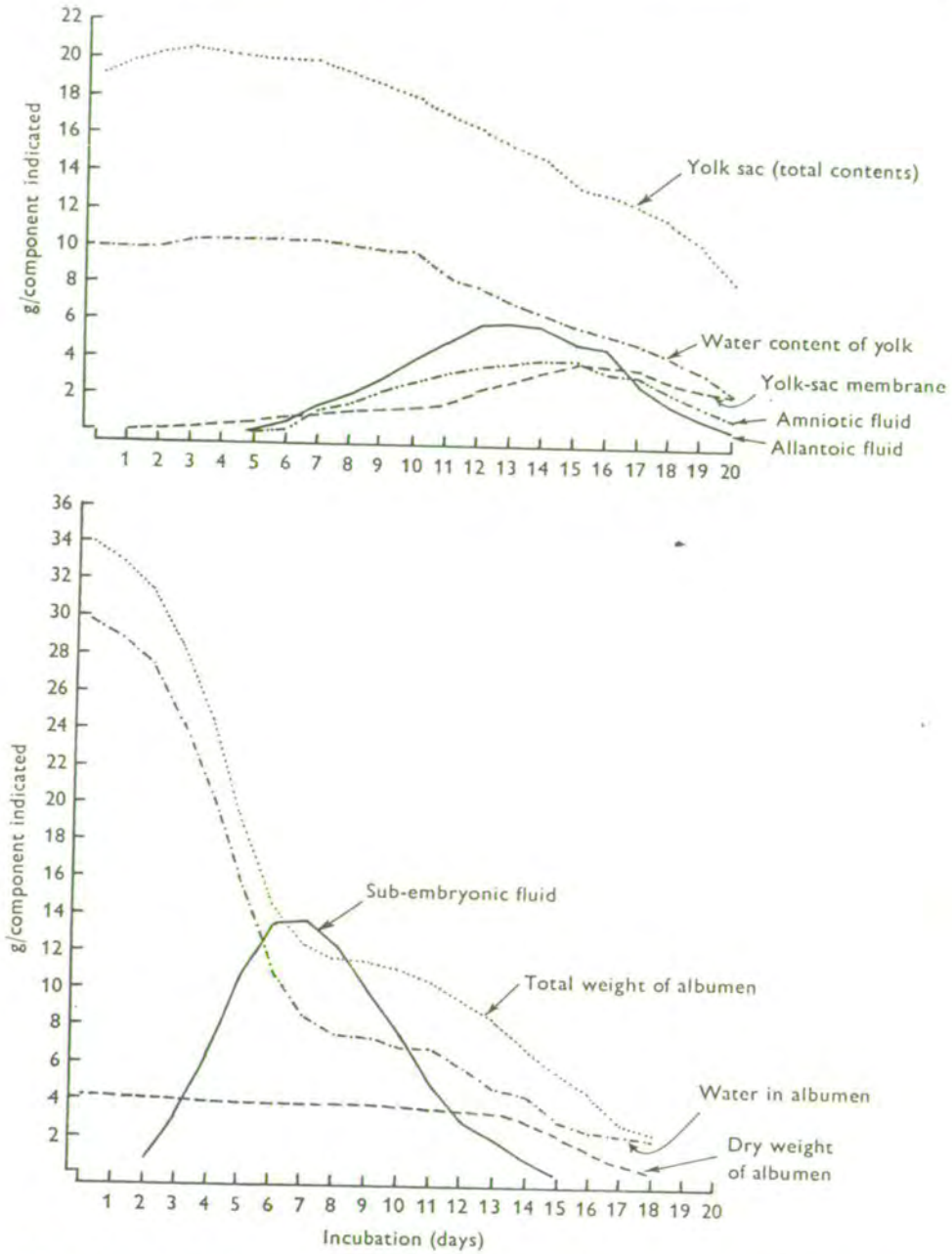
(*d*) *Changes during embryogenesis*

Does the albumen change during development of the embryo so that its bacteriostatic properties are enhanced or diminished? The extraction of water to form the sub-embryonic fluid (Text-fig. 1) results in an increase in the concentration of solutes of the albumen, but some preliminary observations (Board & Halls, 1973*c*) suggest that the water activity of the albumen is not reduced sufficiently to impede the growth of Gram-negative bacteria. It has been shown that the albumen retains its inimical properties during the time that it is held in the amnion (Korotkova, 1957*b*, 1959; Toshkov & Shirova, 1970). Electrophoretic studies (details not given) (Marshall & Deutsch, 1950) and lysozyme assays (Greenfield & Bigland, 1971) support the view that the albumen remains an unfavourable medium for microbial growth at least until it is swallowed by the embryo.

The majority of Gram-negative (hence lysozyme-resistant) bacteria remain viable in albumen *in vitro* (Board & Halls, 1973*c*), providing the temperature of storage is in the range over which the organism can grow (Ayres & Taylor, 1956; Board & Ayres, 1965). This retention of viability by iron-deprived bacteria in albumen is in marked contrast to the behaviour of related bacteria in the serum of freshly drawn vertebrate blood where deprivation renders the cells liable to lysis by complement and, perhaps, lysozyme (Fletcher, 1971). It was noted previously that during embryogenesis the albumen is transferred *via* the amnion to the embryo. As the breakdown of the cell walls of Gram-negative bacteria releases pharmacologically active lipopolysaccharides and lipoproteins, it could be expected that such substances would be toxic to the cells of the embryo, and it is perhaps for this reason that the antimicrobial defence systems do not cause lysis of contaminants of the albumen.

(4) *Course of infection*

The majority of eggs of domestic hens contain no viable organisms at oviposition (Brooks & Taylor, 1955) and they are likely to harbour few organisms even when congenital contamination has occurred (Harry, 1963*a*, *b*). Under commerical conditions of intensive incubation and rearing, pathogens transmitted through the egg



Text-fig. 1. A summary of the changes in the distribution and amount of materials in various parts of the incubated egg of the domestic hen.

(Payne, 1968) can infect chicks (Landauer, 1967). Although such transmission may well occur in nature, it would presumably be important only in birds nesting in flocks where there would be opportunities for the onset of epidemics. Is it in this situation that maternal antibodies provide passive immunity to chicks being reared by parents who are carrying specific pathogens?

The information on rotting tends to be concerned mainly with infertile eggs because of the stimulus provided to research by the need to prevent addling in eggs intended for human consumption. Thus it is pertinent mainly to the situation before the onset of continuous brooding. In practice, three stages can be recognized in the development of a rot: (*a*) contamination and microbial penetration of the shell, (*b*) colonization of the shell membranes, and (*c*) a fulminating infection of the yolk and white without necessarily any macroscopic changes in either.

The shell is assumed to be free of contaminants whilst in the oviduct and hence acquires the first contaminants at or immediately following oviposition (Stuart & McNally, 1943). As contamination is by contact with surfaces in the nest, etc., the micro-organisms tend to be situated at the surface of the shell (Büchli, 1967) and the extent of contamination must be determined by the cleanliness of the egg's environment (Rosser, 1942). With the eggs of domestic hens the level of contamination normally ranges from 10^4–10^7 viable bacteria/shell (Board, Ayres, Kraft & Forsythe, 1964; Board, 1969) and the organisms are derived mainly from the ubiquitous depots of soil and faecal material (Haines, 1938). The available evidence indicates that the micro-organisms remain at the surface of the shell unless some agency promotes their translocation through the pores. In practice, three such agencies have been recognized: (*a*) infiltration of the pores by fungal hyphae, (*b*) flooding of the pores (and hence the passive translocation of micro-organisms) with water drawn in by capillary attraction, and (*c*) the sucking in of contaminated water when a warm egg contracts on cooling.

The rate and extent of the growth of moulds on the surface of the hen's egg is determined by the relative humidity of the atmosphere, there being good growth at 100% R.H., meagre growth at 94–90% and no growth at 88% at a storage temperature of 30 °F (Sharp & Stewart, 1936). From eggs which were intended for human consumption many species of moulds have been isolated (Weston & Halnan, 1928; Lorah, Funk & Forward, 1954), whereas *Aspergillus fumigatus* is of importance in eggs during incubation.

In the studies on infection of the shell by moulds, little attention has been given to the source of nutrients used by the moulds. It can be assumed that bad soiling of the shell would provide nutrients and it was noted (Board & Halls, 1973*a*) that the cuticle was digested by streptomyces growing on the surface of eggs kept in a saturated atmosphere. The digestion of the cuticle removed the barrier to the invasion of the pores with carbon black. In a recent survey (Seviour, Sykes & Board, 1972) it was noted that the eggs of a great number of species of ducks, geese, etc. taken from brooding bantams had a high incidence of contamination with moulds; there were coloured patches in the shell membranes and/or lumps of gelatinous albumen adhering to the membranes. In all cases, however, the albumen was heavily infected with bacteria and it was not possible to determine whether or not their presence was due exclusively to

translocation on hyphae. In any case, it would seem that eggs lying in wet nests, such as those of waterfowl or ground-nesting species, would be at risk both to infection by mould and the contamination of the albumen with bacteria (Zagaevsky & Lutikova, 1944). As yet, however, there is no evidence of the means whereby mould growth is controlled; are the moulds rubbed from the surface of the shell during egg turning and/or do the preening oils have fungicidal properties?

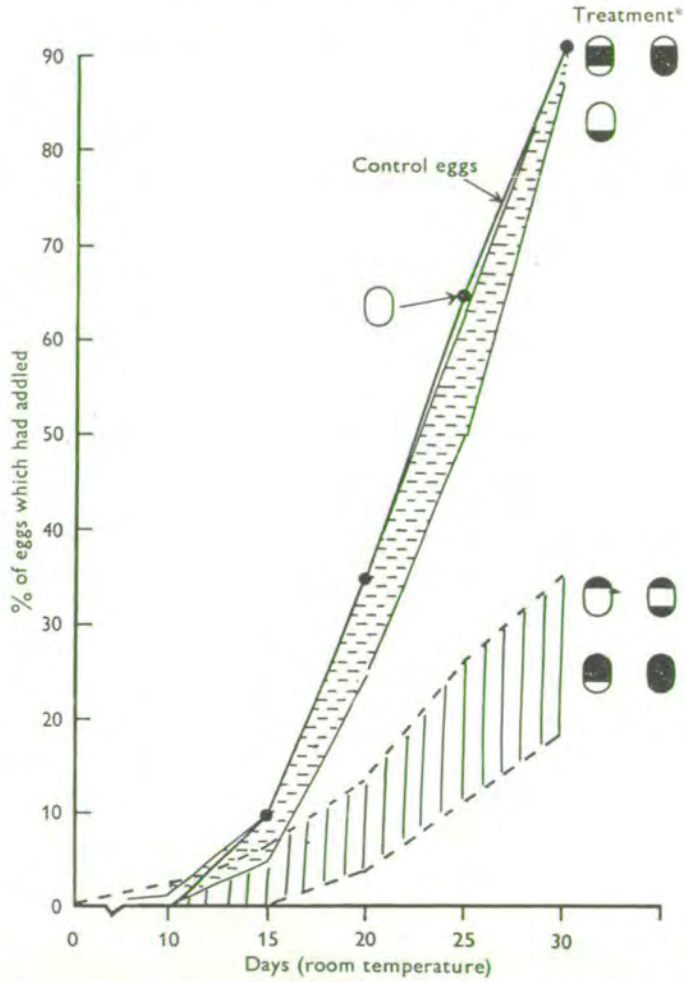
The possibility of bacteria being translocated in the water drawn into pores by capillarity was suggested by the work of Haines & Moran (1940) but the studies of washed eggs by Büchli (1967) and carbon black penetration by Board & Halls (1973*a*) suggest that this process plays only a minor role in the infection of the contents of the eggs of domestic hens. This is a reflexion presumably of the water-repellency of the shell of avian eggs. When a warm egg cools in cold water, the contents contract more than the shell and the pressure differential generates suction, whereby the water-resistance of the shell is overcome and water is pulled along the pore canals (Haines & Moran, 1940). Since the recognition of this phenomenon, the dipping of warm eggs in chilled suspensions of bacteria has been used experimentally to initiate infection of the contents of the eggs of domestic hens. From the evidence obtained with chilled suspensions of carbon black (Board & Halls, 1973*a*), it appears that only a very small proportion of pores are flooded in shells which have cuticle both at the surface of the shell and within the pores. With eggs lacking cuticles, such as pigeon eggs, a large number of pores are flooded. When the rate and/or extent of rotting is used as an index of penetration of the shell with bacteria, the following have been shown to be important: (*a*) the temperature differential between the egg and the bacterial suspension, the incidence of rotting being directly proportional to the temperature difference in the range 6–21° (Lorenz, Starr, Starr & Ogasawara, 1952; Brant & Starr, 1962); (*b*) the number of organisms in the suspension (Lorenz *et al.*, 1952; Stokes, Osborne & Bayne, 1956; Hartung & Stadelman, 1963); (*c*) the duration of immersion (Brant & Starr, 1962; Hartung & Stadelman, 1963); (*d*) the thickness of the shell (Orel, 1959). and (*e*) the treatment of the shell before immersion; damage to the cuticle by physical or chemical means increases the extent of penetration of the shell (Board & Halls, 1973*a*). With the temperature differential, it can be assumed that the greater the temperature difference between the egg and water, the greater will be the amount of water and hence the number of organisms pulled through the pore canal. Moreover, the heavier the inoculum, the greater the opportunity for the coincidence of bacteria and imperfectly sealed pores.

Although the available evidence comes from studies of the infertile eggs of domestic hens, it does permit the identification of conditions in the nest which could be expected to promote the translocation of bacteria across the shell. Of the agencies considered, suction generated by temperature fluctuation may well be of major importance, especially with eggs enclosed in cuticle and laid at the beginning of a clutch. With cuticle-less eggs raindrops might well cause micro-organisms to be forced into the pores. It has been suggested (Ferdinandov, 1944) that bacteria are drawn into the egg when it cools after oviposition, and it has been shown (Gordon & Tucker, 1954) that there is a high incidence of rotting in eggs laid in nests contaminated deliberately with *Pseudo-*

monas sp. Dirty nesting materials and bad soiling of the shell are known to be associated with high incidences of yolk-sac infections in commercial hatcheries. It may be that heavy contamination of the shell promotes infection of the albumen with bacteria but these remain quiescent, through Fe^{3+} deprivation or because they are in the spore stage, until the mixing of yolk and albumen on the twelfth day of incubation provides a favourable environment for their growth.

When immersion of warm eggs in cold suspensions of bacteria has been used to initiate rotting of eggs, there is a lag of upwards of 10–15 days between the challenge of the fresh eggs and the onset of rotting, as determined by the detection of viable organisms or by overt changes of the yolk and white, which can be correlated with enzyme(s) of the test bacterium (Zagaevsky & Lutikova, 1944; Gillespie & Scott, 1950; Bigland & Papas, 1953; Miller & Crawford, 1953; Stokes *et al.*, 1956; Orel, 1959; Fromm & Monroe, 1960; Garibaldi & Bayne, 1960; Rizk, Ayres & Kraft, 1966; Büchli, 1967; Vadehra *et al.*, 1970*a*).

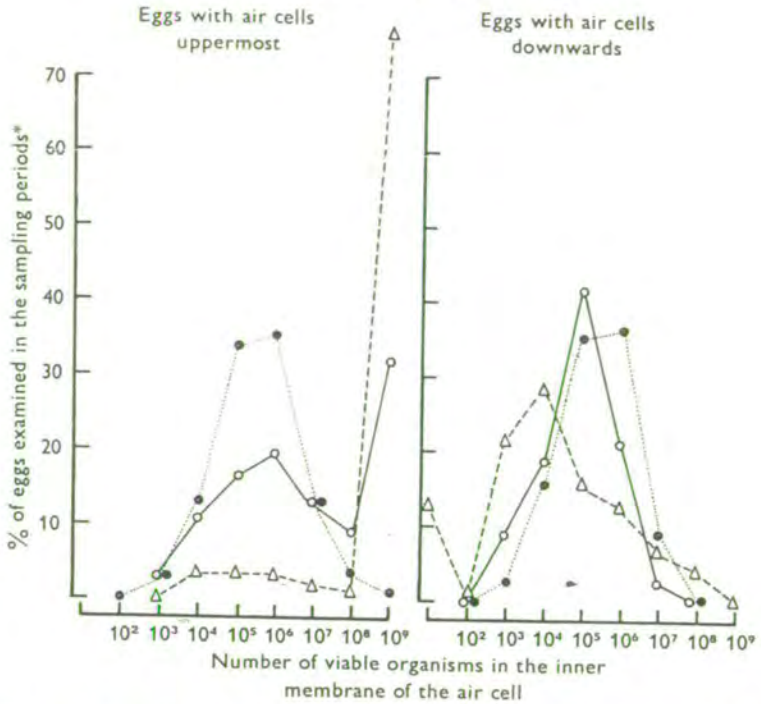
As a lag of the same magnitude was noted by Brooks (1960) when he placed a heavy inoculum on the inner membrane of the air cell, it cannot be attributed to the slow movement of bacteria across the cone layer of the shell. Brooks kept the eggs with the air cells uppermost at 30 °C. Ever since Almquist & Holst (1931) demonstrated that dyes penetrate the broad end more easily than the pointed pole of the shell, there has been a tendency to consider that the former is the more prone to bacterial infection. Such a contention received support from the observation (Lorenz *et al.*, 1952) that a high incidence of rotting resulted from the application of soil to the broad pole of eggs. Likewise the observations (Vadehra *et al.*, 1970*b*) – summarized in Text-fig. 2 – suggest at first sight that different parts of the shell offer different resistances to bacterial penetration. The results summarized in Text-figs. 3 and 4 show, however, that the fate of bacteria present in the shell membranes of eggs maintained at room temperature is dependent upon the location of the infected membrane within the egg. With the air cell uppermost for 0–9 days, there was a normal distribution in the level of contamination of the inner membranes of the air cell (Text-fig. 3) and a relatively low level of contamination of the albumen (Text-fig. 4). On further incubation there was bimodal distribution in the sizes of the populations in the inner membrane of the air cell, and the second peak was associated with gross contamination and rotting of the whole contents of the egg. When the infected air cell was kept downwards, a unimodal distribution of population sizes in this membrane was found throughout incubation and there were only slow increases in the incidence and extent of contamination of the albumen. This evidence indicates that bacteria retained by the shell membranes or those which gain access to the albumen remain quiescent until some change in the egg induces their multiplication. It has been suggested (Board, 1964) that in eggs held at room temperature it is the union of the yolk and shell membranes which induces multiplication – the union allows the contaminants to exploit the nutrients of the yolk in a situation which is not influenced by the albumen. Assuming that Vadehra *et al.* (1970*b*) kept their eggs with the air cells uppermost, it can be deduced from the data in Text-fig. 2 that it was the location of the nidus of infection relative to the movement of the yolk and air cell and not the actual site which gave such marked differences in the



Text-fig. 2. The influence of the site of challenge on the rate and incidence of rotting of the eggs of domestic hens. The shells were covered with wax in the places shown (black) on the symbols on the right-hand side before being challenged with bacteria. Data taken from Vadehra, Baker & Naylor (1970*b*).

incidence and rate of rotting in eggs which had selected portions of the shell sealed with wax. This implies that the viscosity and integrity of the albuminous sac play a cardinal role in protecting the yolk from bacterial infection. Such an interpretation is in accord with the observations (Zagaevsky & Lutikova, 1944; Ayres & Taylor, 1956) that rotting is rapidly established when bacteria are placed in the yolk. This evidence indicates that with eggs laid at the beginning of a large clutch infection of the shell membranes, if it were to occur, would be mostly confined to those membranes and, to judge from the observations of Brooks (1960) and those summarized in Text-figs. 3 and 4, even contaminants of the albumen would remain quiescent.

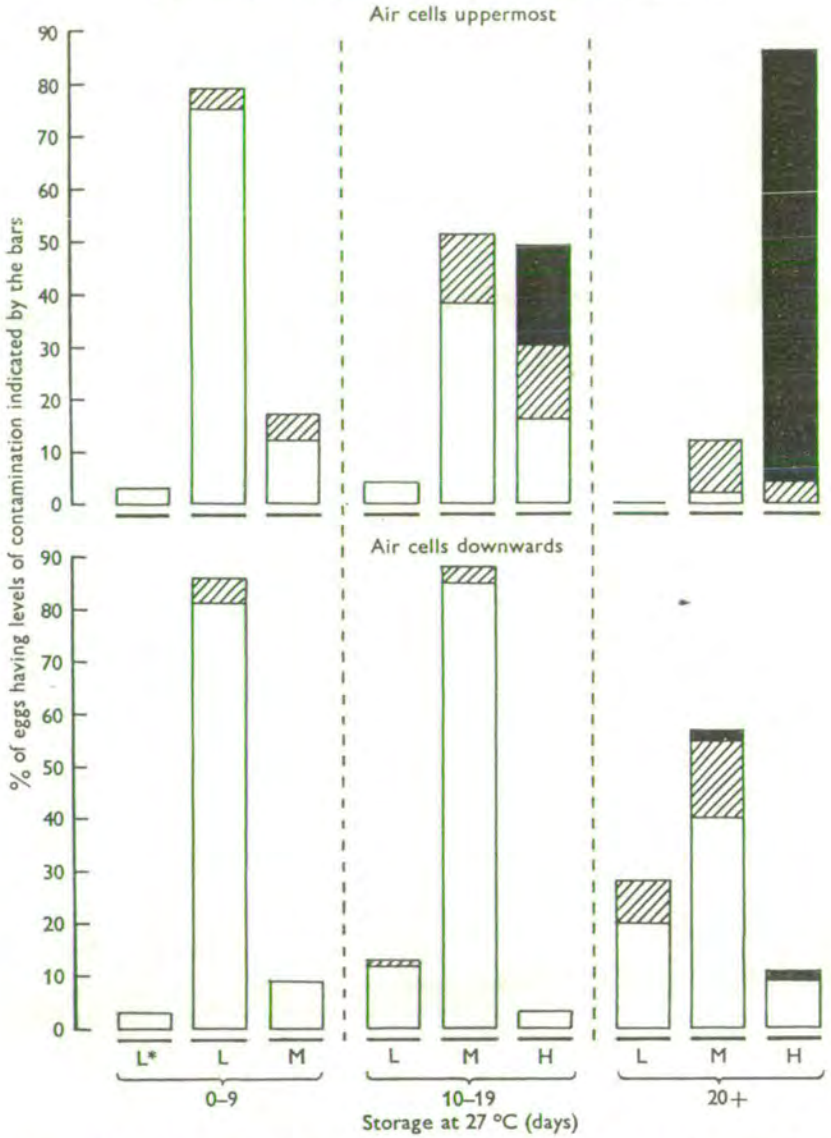
In the past three years, one of us has failed to establish rot in eggs inoculated



Text-fig. 3. The influence of the position (either uppermost or lowermost) of infected air sac on the growth of bacteria. The inner membrane of the air cell was infected with *ca.* 10^3 bacteria; the number of bacteria present in the air cell was determined in the periods 0–9 (closed circle), 10–19 (open circles) and 20+ days (open triangles). Number of eggs examined, 619 (unpublished data of R. G. Board). Eggs stored at 27 °C.

in the air cell with 10^3–10^4 bacteria and maintained, either immediately or after storage at room temperature for 3–4 days, in an incubator at 39·5 °C. A wide range of organisms, all of which came from rotten eggs (Seviour *et al.*, 1972), were used in these studies. The infection was confined to the shell membranes. There was no evidence of multiplication even when eggs were unturned, and indeed, a suggestion that the organisms died rapidly when the extra-embryonic membranes grew along the inner surface of the infected air-cell membrane. Healthy chicks were hatched from such eggs. Preparation of an inoculum in extracts of soil or faeces did result in multiplication of the organisms in the shell membranes, but the extent was less at 39·5 °C than that obtained in eggs inoculated in the same manner and held at 27 °C (Board, 1964). Moreover, neither the soil nor the faecal extract induced rotting. This difference between eggs held at 39·5 °C and at 15–30 °C cannot be interpreted in terms of temperature accentuating the antimicrobial properties of the albumen. It has been shown (Board & Halls, 1973*c*) that the behaviour of *E. coli* in albumen held at 39·5 °C is essentially the same as that of the same organism in albumen at room temperature (Seviour & Board, 1972).

Thus the available evidence indicates that the shell membranes, through their inhibition of microbial growth, play a major role in preventing the rotting of eggs



Text-fig. 4. Influence of position of infected air cell on the incidence and extent of contamination of the albumen (L, light, M, medium, H, heavy). Experimental details noted in Text-fig. 3. Level of contamination of inner membrane of air cell, light (open bars), medium (hatched bars) and heavy (solid bars).

during incubation and that the albumen is a second line of defence. As yet there is no evidence, apart from the speculation that hyaluronic acid may be involved (Osuoji, 1971), of the means whereby the membranes inhibit the growth of bacteria, and future work might well be directed to a study of the biological properties of the glycoprotein mantle on the keratin fibres of these membranes.

III. THE NEONATAL BIRD

The class Aves is such a heterogeneous group that it is impossible to make general statements that will apply to all the 8580 species living in the world today. They have evolved to fill almost every ecological niche on the face of the earth and in doing so have no doubt developed different methods of combating microbial invasion. It seems certain that the altricial species which are born naked at an early stage of development will be less mature immunologically than the precocial species which are born with feathers and at an advanced stage of development. Most of the work recorded on the immunology and defence mechanisms of birds refers to galliform species. This knowledge, which derives mainly from studies on chickens with an incubation period of 21 days, is unlikely to apply without modification to the passerine species, some of which hatch after about 11 days. These latter birds, because they are less mature immunologically, may well be more dependent on non-specific mechanisms for their defence against micro-organisms. On hatching both altricial and precocial birds lose all the mechanical and chemical barriers that surrounded them during embryogenesis. It is therefore important that the immune machinery of the newly hatched chick becomes fully functional as soon as possible to protect it against the very considerable bacteriological assault which it faces at this stage of its life. Immune responses by their very nature take time to become effective and there may be an immunological hiatus immediately after hatching. Immunocompetent cells for humoral immunity are first detectable at 4 days after hatching (Toivanen, Toivanen & Good, 1972). Although the synthesis of high molecular weight antibody (IgM) is initiated soon after hatching it takes several weeks for it to reach adult levels (Merkenschlager *et al.*, 1967). Moreover the level of the low molecular weight antibody (IgG) falls for about 2 weeks after birth. The latter is composed partly of actively produced antibody, as is shown by the rate of decline being faster in germ-free chickens (Merkenschlager *et al.*, 1967), and partly of antibody passively acquired from the yolk sac. The range of antibodies in the yolk is restricted to those raised in response to antigens which the hen has experienced. While this may be adequate under natural conditions where the bacteriological environment is similar for both hen and chick, under modern rearing conditions the environment of the hen producing the egg may be very different from that of the incubator and brooder room in which the young chick is hatched and reared. This difference may also be of importance where artificial incubation or foster mothers are used to brood the eggs of threatened species. In the present state of knowledge it is difficult to assess the extent to which cell-mediated immunity is protective during this neonatal period. If the immune response is inadequate to protect the chicken in the first few days of life, what factors are responsible for the increase in resistance to infection which undoubtedly occurs?

(1) *Physical barriers*

The first line of defence presented to a potential pathogen is the epithelium of the chicken. The skin and mucous membranes must be breached if the invasion of the host is to proceed. Of course these linings are seldom complete, and few animals are

entirely free from scratches on the skin or imperfections in their mucous membranes. Overcrowding such as occurs in broiler houses, or naturally in colonies of terns, increases the risk of the skin being scratched. Abrasive particles such as hard grains in graminivorous birds or pieces of bone in birds of prey may produce minute ruptures of the gut lining which enable bacteria to penetrate beyond the epithelial surface. Hamdy, Brown & McCarthy (1963) claim that *Escherichia coli* and *Staphylococcus aureus* can enter bruised chicken tissues by penetrating skin, air sacs and intestine; even unbroken epithelial layers may not be proof against bacteria. The problem of containment is probably greatest in the intestine, where only the gut mucosa stands between a large complex microflora and the rest of the body. Fuller & Jayne-Williams (1968, 1970*a*) showed that even in healthy chickens intestinal bacteria such as streptococci, coliform organisms and anaerobic bacteria could be recovered from the yolk sac, peritoneum and liver. These bacteria, when fed to germ-free chickens, appeared in the same three sites. This translocation from the intestine to the liver reached a peak at about 3 days and declined rapidly, until at 10 days it was no longer demonstrable.

Presumably the bacteria are carried through the epithelial cells in pinocytotic vacuoles similar to those which transport lipid and proteins. Because the chicken fails to discriminate between what it does and does not want, it occasionally picks up bacterial cells. The newly hatched chicken cannot deal with this infiltration and bacteria appear in the liver. As the chicken gets older its resistance increases and thus translocation appears to stop. However, it is suggested that bacteria may continue to escape from the gut but they fail to be detected in the liver because they are rapidly killed by the host defences.

When chicken eggs are treated with a solution of testosterone propionate the development of the bursa of Fabricius (a lymphoid organ lying on the dorsal surface of the cloaca) is suppressed and antibody formation is adversely affected. Warner, Szenberg & Burnet (1962) found that chickens whose IgG response was impaired in this way often died of bacteraemias caused by *Strep. faecalis* or *E. coli*. The implication was that as a result of the lowered capacity for IgG response the translocated organisms from the gut had been allowed to multiply and cause fatal bacteraemias. Fuller & Jayne-Williams (1970*b*) were unable to repeat these results. Their bursectomized birds remained healthy and grew as well as the controls. Even when the bursectomized birds were injected intravenously with a suspension of organisms prepared from chicken intestinal contents, only 1 out of 12 birds died. This and other evidence suggest that the development of resistance to bacterial invasion from the gut is not antibody-dependent (Fuller & Jayne-Williams, 1970*a*, *b*). However, during this period three things are happening which make the reticulo-endothelial system more efficient: the number of Kupffer cells in the liver increases twofold; the large amount of lipid present in the liver of the day-old bird declines (Pl. 1A, B) and the body temperature of the chicken rises from 38 to 41 °C. The lipid present in the newly hatched bird may have a blockading effect on the reticulo-endothelial system so that its removal during the first few days of life might allow the Kupffer cells to operate more effectively. Similarly the rise in temperature increases the efficiency of phagocytosis. This aspect is discussed more fully in a later section (p. 38).

Thus the day-old bird deals relatively ineffectively with bacteria which migrate from the gut because not only has it fewer phagocytic cells but those that it has are working below their maximum potential. Any factors which tend to depress the resistance of the bird (e.g. low brooding temperatures) may lead to an upsurge of disease caused by translocated intestinal bacteria. Diseases in poultry such as endocarditis caused by *Strep. faecalis* and yolk-sac infections caused by *E. coli* may be due to migration of bacteria from the gut into the tissues of a chicken whose resistance is low.

Peristalsis is a physical factor which tends to work to the disadvantage of bacteria trying to colonize the intestinal tract. The continuous movement of food along the gut means that if organisms are to colonize that site successfully their rate of multiplication must be greater than the rate at which they are diluted by peristalsis. In bats and some birds the transit time is so short that bacteria hardly have time to multiply and the intestinal microflora is consequently comparatively simple. Some organisms counter the effects of peristalsis by multiplying rapidly, others slow down their rate of removal by adhering to the gut wall. However there must be many organisms (including pathogens) which, because they can neither grow fast enough nor attach themselves to the gut wall, are swept straight through the intestinal tract.

(2) *Phagocytosis*

In common with the mammalian foetus, the avian embryo goes through stages in ontogeny which recapitulate those which have occurred during phylogenetic development. Thus, in the early stages of embryonic development the primitive situation exists and phagocytic cells are found in many different tissues. As development progresses and the reticulo-endothelial organs become more highly organized they gradually take over the function of phagocytosis. By the time the chicken hatches, virtually all significant phagocytosis is restricted to the liver, spleen and lungs. These three organs continue to function as the major sites of phagocytosis throughout the rest of the bird's life.

The detailed sequence of events on which this general statement is based has been followed by means of thorotrast, colloidal silver (Kent, 1961) or carbon particles (Nicol, Cox, Bilbey & Strachen, 1962; Mizejewski & Ramm, 1969). The more detailed work of Mizejewski & Ramm (1969) showed that carbon uptake by the 31 organs and tissues studied could be categorized into seven groups. In the early stages of development the heaviest deposition was in the heart and blood vessels. This gradually diminished during embryogenesis. In the thyroid, parathyroid and ultimobranchial bodies the deposition was initially slight, then moderate in mid-incubation (10 days) and again slight during the terminal period. The bone marrow, hypophysis, mesonephros and suprarenals showed a steady increase in phagocytosis. The pancreas and gonads started with slight deposition which decreased to a trace just before the time of hatching. Phagocytosis in the liver, spleen and lung all increased steadily, although at different rates, to give heavy carbon deposits at hatching. The mesonephros was considered to be a special case related to this last group since phagocytosis increased in it until vascular degeneration set in. In the other organs and tissues examined only trace amounts of carbon were detected.

Studies as detailed as those of Mizejewski & Ramm (1969) have not been made with living organisms in place of inert particles. This is unfortunate since it is well known that the type of particle presented to the phagocyte can influence the course of ingestion. The basis of this variation appears to be partly dependent on opsonins, since even inert particles like bentonite require opsonization (Potter & Stollerman, 1961) before maximum ingestion can take place. However, the degree of opsonization required may depend on the physiological state of the micro-organism being phagocytosed. To some extent experimental studies are unnatural in the sense that the micro-organism presented for phagocytosis is derived from a culture grown in a nutrient medium, whereas in practice the phagocytes may well be confronted by a debilitated bacterial cell which does not require opsonization. Without further bacteriological studies it is difficult to establish whether the phagocytosis of carbon particles observed in embryonic tissues is truly indicative of a protective effect. If the ingestive capacity is not matched by functional bactericidal mechanisms within the cell then phagocytosis is likely to benefit the bacterium by protecting it from the inimical environment outside the cell. Indeed, Buddingh & Polk (1939) were of the opinion that meningococci survive and even multiply inside the phagocytes of the chick embryo and Goodpasture & Anderson (1937) considered that *Strep. viridans*, *Aerobacter aerogenes*, *Sal. typhi*, *Brucella abortus* and *Mycobacterium tuberculosus avium* all benefited from an intracellular habitat.

Even in mammals, events after the engulfment of bacteria are poorly understood and many different mechanisms have been postulated to account for the killing of intracellular bacteria (Hirsch, 1972). For example, cationic proteins, lysozyme, myeloperoxidase, hydrogen peroxide, lactic acid and histones have all been shown to reach inhibitory concentrations within the cell. Few such studies have been made on chicken cells although the ability of chicken phagocytes to kill engulfed *Sal. gallinarum* (Karthigasu, Reade & Jenkin, 1965), *Staph. albus*, *Serratia marcescens*, *E. coli* and *Candida albicans* (Brune, Leffel & Spitznagel, 1972) has been demonstrated. In chicken heterophils, lysosome-like structures have been demonstrated by histochemical techniques (Topp & Carlson, 1972) and seen in electron micrographs (Dhingra, Parrish & Venzka, 1969) and these granules have been observed to lyse after the cells have engulfed bacteria (Hirsch, 1962). However, quail and chicken leucocytes are apparently deficient in those enzymes which are traditionally associated with the intracellular killing of bacteria (Atwal & MacFarland, 1966; Brune *et al.*, 1972).

In the main, our knowledge of phagocytosis in the chicken comes from experiments *in vivo* in which the rate of disappearance of bacteria from the blood is taken as evidence of ingestion and the rate of decline in numbers in the liver and spleen as an index of bactericidal capacity. To the extent that phagocytosis shifts from the egg membranes to the liver, the results with carbon particles have been reproduced with ^{32}P-labelled *E. coli*. The rate of phagocytosis in the embryo depends on the type of organism used; rough strains of *E. coli* are phagocytosed more readily than smooth strains. This difference can be correlated with the presence of opsonins for rough strains but not for smooth strains (Karthigasu & Jenkin, 1963). The neonatal chicken can remove smooth and rough bacteria with equal efficiency due, it has been suggested,

to the absorption of macroglobulin from the gut after hatching (Karthigasu, Jenkin & Turner, 1964). Not everyone would agree, however, that IgM antibody is secreted into the yolk (Malkinson, 1965) and it seems very doubtful whether yolk is transferred along the yolk stalk to the intestine (Lillie, 1952). Moreover, studies using polyvinyl pyrrolidone with a mean molecular weight of 160000 (i.e. similar to IgG but smaller than IgM) have shown that it is not taken up by the small intestine of young chicks. The intestine shows none of the histological characteristics normally associated with macromolecular uptake (Clarke & Hardy, 1970). Thus even if IgM antibody reached the gut it would probably not be absorbed. It seems more likely that it would be absorbed directly from the yolk sac (Solomon, 1971). When the hens are immunized with smooth strains they produce IgG antibody, which is transferred to the embryo *via* the yolk; phagocytosis of smooth bacteria is then possible.

The relevance of opsonins to neonatal resistance is still questionable. They are not important in resistance to infections by *Sal. gallinarum* (Karthigasu *et al.*, 1964; Solomon, 1968*a*). Instead, it is suggested that the increase in resistance is due to increased bactericidal capacity of the intracellular environment (Karthigasu *et al.*, 1965).

It is usually assumed that phagocytosis is performed by macrophages and polymorphonuclear leucocytes. However, a phagocytic function has been recently ascribed to chicken thrombocytes (Carlson, Sweeney & Tokaryk, 1968) and to chicken and pheasant erythrocytes (Prunesco & Prunesco, 1972). If this is confirmed the role of these cells in resistance may have to be considered.

(3) *Serum factors*

Little is known about the non-specific serum factors of the chicken. Even complement, which is very well characterized in the mammals, has received comparatively little attention especially in the context of bacterial inhibition. It is certainly present in chicken serum and acts in concert with specific antibody to lyse cells. It resembles guinea-pig complement in its heat lability and inactivation by EDTA but cannot be replaced by mammalian complement in lytic systems containing fowl antibody (Rose & Orlans, 1962). Complement is detectable in 18-day-old embryos (Warren & Borsos, 1959).

Complement-dependent bactericidal activity for *Sal. gallinarum* can be detected in serum of day-old chicks (Solomon, 1968*b*) but there appears to be no correlation between titre of bactericidal activity and resistance to *Sal. gallinarum* infection. By contrast Collins (1967) could not detect bactericidal antibody for this organism in chickens of any age. These conflicting results may reflect the labile nature of this component.

Non-antibody bactericidins for *Staph. aureus* (Cybulska & Jeljaszewicz, 1966) and *Sal. gallinarum* (Solomon, 1968*b*) can also be demonstrated in chicken serum but they do not appear to be involved in resistance to infection with these two organisms.

The chicken is naturally resistant to pneumococcal infection. This resistance is not due to antibody, but a serum factor is detectable which when transferred to mice will protect them (Andrews & McKinnon, 1961). However, the natural resistance of the

pigeon to pneumococcal infection was considered by Bateman & Rowley (1969) to be due to natural antibody. The extent to which factors like lysozyme and conalbumin are carried over from the egg into the chicken serum would merit further study. Transferrin, which like conalbumin is an iron-binding glycoprotein, is found in chicken serum but differs from conalbumin in that it has a different carbohydrate moiety (Williams, 1962). The inhibitory effects of conalbumin have been discussed in a previous section (see p. 22) and the involvement of transferrin in bactericidal properties of both equine and human serum has been demonstrated by Rogers (1967). The level of serum transferrin is increased in bursectomized birds (Morgan & Glick, 1972). Could this be an attempt on the part of the chicken to compensate for the depressed IgG levels in these birds?

The phenomenon of viral interference has been known for over 30 years but it was not until 1957 that Isaacs & Lindenmann described interferon, which is responsible for the inhibition of a wide range of viruses following viral infection. There are many different interferons, their nature depending on the animal species responding, the type of inducing agent used, the site of induction and the time after induction. The interferon of the chick embryo is the most widely studied and the best characterized. It is a protein containing a small amount of carbohydrate. It has a molecular weight of 25000–40000, is stable between pH 2 and pH 10 and at 65–70 °C for 1 hour.

Although chick embryos and neonates are immunologically immature their interferon response is as good as that of an adult bird. Interferon production therefore seems to have enormous potential as a protective mechanism against virus infection during the embryonic and neonatal period. Certainly an increasing natural resistance to viruses can be demonstrated in the chick embryo during its development (McClain & Modin, 1955; Morahan & Grossberg, 1970*a*) and the suggestion (Baron & Isaacs, 1961) that this type of resistance may be due to increased ability of the older embryonic tissues to produce interferon has received some support from the work of Morahan & Grossberg (1970*b*) who have shown that at least part of the age-related anti-viral resistance of the chick embryo is due to interferon.

Because the interferon response is effective within hours after stimulation, attempts have been made to raise the level of interferon in the newly hatched chicks in order to protect them until the immune response becomes adequate at 1–2 weeks. Bankowski & Kaleta (1972) showed that the strains of Newcastle-disease virus used for vaccines are poor inducers of interferon, and concluded that the best combination for producing maximum protection was a highly immunogenic vaccine and a potent non-viral interferon inducer, e.g. certain complexed synthetic double-stranded polynucleotides.

(4) *Tissue localization*

It is well known that some organisms show a predilection for certain tissues and grow almost exclusively in those sites. One of the best examples of this phenomenon is contagious abortion in cattle. In this disease the bacteria (*Br. abortus*) are concentrated in the foetal cotyledons (Smith *et al.*, 1961) because the latter have a high erythritol content (Smith *et al.*, 1962). The reason for localization is not always so clear-cut and

it is often difficult to separate the resistance of a tissue that depends on the absence of a necessary metabolite from that dependent on the presence of antibacterial factors.

The possibility that changes in the nutrient environment of the cells of the embryonic and neonatal chick might make conditions for the growth of bacteria less favourable has not been explored to any great extent. Goodpasture & Anderson (1937) studied the course of infection of seven species of bacteria in 12- to 14-day-old embryos. They found that the tissue localization of these organisms varied greatly. In their work a heavy inoculum in nutrient broth was applied to the extra-embryonic membranes, and thus the tests can be considered to be highly artificial because of the findings, discussed earlier, that in the whole egg under natural conditions only nutritionally deprived organisms would normally challenge such membranes.

The same criticism cannot be made of the work of Bigland (1972). He used eggs from a flock naturally infected with *Mycoplasma meleagridis* and found that in the embryo the organisms were confined almost exclusively to those tissues which later become involved in respiration. He considered that this growth in the embryonic respiratory tissue indicated that these sites were biochemically suited to the needs of *M. meleagridis*.

One well-documented example does occur in the chicken. Newly hatched chickens suffer from a disease of the yolk sac (so-called Mushy Chick Disease) which may be caused by a variety of different bacteria many of which (e.g. *B. cereus*, *Staph. aureus* and *Clostridium sporogenes*) produce enzymes that enable them to utilize the yolk lipids (Harry, 1957). The amount of multiplication that occurs in the yolk is proportional to the amount of decomposition of the yolk. Moreover, *B. cereus* is not pathogenic for the chicken or egg except when inoculated into the yolk. There appears to be a delicate balance between inhibition and stimulation in the yolk, because many yolks which would not support the growth of *B. cereus* did so when diluted with an equal volume of water. Thus we have the situation where an organism is completely dependent on the presence of a particular organ for its pathogenicity.

(5) *Body temperature*

Until the chicken embryo pierces the air sac on the 20th day of incubation it behaves like a cold-blooded animal. At this time pulmonary respiration starts and there is a gradual change to a homeothermic state. During the next 10 days the body temperature of the chicken rises by 3 °C. In other species (e.g. cliff swallows) this period of adjustment may last as long as 21 days. During the period of rising body temperature in the chicken there is an increasing resistance to *Salmonella*. Scholes & Hutt (1942) attempted to relate body temperature to resistance and, as well as proving that neonatal resistance to salmonellosis increased with increasing body temperature, they also showed that artificially lowering the body temperature, by brooding at 28° or by administration of sodium amytal, lowered the resistance. Resistance could again be increased if the body temperature was raised by high brooder temperatures. Differences in resistance between different breeds and even different individuals of the same breed tended to be related to body temperature. The authors did not attribute

resistance to a direct effect of temperature on the bacteria but suggested that it resulted from an increase in the efficiency of some unspecified defence mechanism of the host. Bell (1949) extended these studies to determine the particular mechanism involved. He confirmed many of the observations of Scholes & Hutt (1942) and showed also that the phagocytes of resistant birds were more bactericidal than those of susceptible birds and that both resistant and susceptible phagocytes exhibited greater ingestion with increasing temperature.

While the elevated temperature associated with resistance is insufficient in itself to kill *Salmonella* it is possible that their metabolism is adversely affected, thus making them more susceptible to phagocytosis or other antibacterial mechanisms in the host. It is known for example that when the temperature of a culture of *E. coli* in minimal medium is shifted from 37 to 45 °C the growth rate immediately slows down. The original rate can be restored by supplying methionine (Ron & Davis, 1971). Thus in the chicken, nutrients which may be adequate for bacterial growth at 38 °C (i.e. the body temperature of the newly hatched bird) may be inadequate when the body temperature rises to 41 °C after the first few days of life.

Seto (1972) studied the effect of temperature on antibody production by chicken immunocytes. Blood from chickens that had been stimulated with mouse erythrocytes was injected into 14-day-old embryos which were then incubated at 38 or 41 °C. It was found that antibody production was accelerated at the higher temperature. Seto concluded: "The avian immune system is keyed to function optimally at a relatively high body temperature and is less efficient at lower temperatures."

(6) *Intraluminal intestinal factors*

In 1907 Metchnikoff noted the bactericidal capacity of the gut of certain birds. Describing one of his experiments he wrote: "I have investigated the case of ravens which I fed on flesh which was putrid and swarming with microbes. The droppings contained very few bacteria and it was specially remarkable that the intestines had not the slightest smell of putrefaction." He did not speculate as to the mechanism of this phenomenon but since then it has become clear that many factors may act to control the microflora of the gut.

On the 11th day of incubation of the chick the membranes between the yolk and the albumen and between the albumen and amniotic fluid break down. It has been suggested by Kramer & Cho (1970) that this is important for the transfer of antibody from the yolk to the intestine of the embryo. However, it may be also important as a means of transferring albumen (containing conalbumin and lysozyme) into the embryonic gut and providing the newly hatched chick with a supply of these antibacterial factors. Inhibitory levels of lysozyme are found in the embryonic caecum and cloaca (Greenfield & Bigland, 1971) and in extracts made from the pancreas and anterior small intestine of the chicken (Prince, 1971). Prince & Garren (1966) found that although 60% of the birds challenged with *Sal. gallinarum* survived they did not produce agglutinins. They did, however, have bactericidal levels of lysozyme in the gut. The levels were higher in the resistant White Leghorn breed than in the sus-

ceptible Rhode Island Red breed. Moreover, the lysozyme in the Rhode Island Reds was depleted more rapidly after *Sal. gallinarum* infection (Prince, 1967). It was concluded that lysozyme might not be the only factor involved; it might, for example, be acting synergically with trypsin. Certainly the conditions in the intestinal tract are suitable for lysozyme activity, and while the lysozyme may not eliminate all the salmonellae from the gut, those moving into the tissues would perhaps be more susceptible to phagocytosis and intracellular killing.

By the time the chicken hatches, the proventriculus is already secreting acid (Toner, 1965) and producing a pH low enough to be inimical to most bacteria. Studies *in vitro* in which the pH of the gizzard was reproduced with buffer solutions showed that a pH of 2·6 for 90 min. was bactericidal for *Sal. seftenberg*, *Sal. thompson* and *Sal. typhimurium* (Cox, Davis, Watts & Colmer, 1972). The pH of the proventriculus and gizzard, which can be as low as 1·5 (Herpol & van Grembergen, 1961), would seem to present a formidable obstacle to any organism attempting to colonize the lower gut. Values of pH between 2 and 3 have also been recorded for the gizzards of the pigeon, pheasant, duck and turkey (Farner, 1942) and between 1·0 and 1·5 for that of the white-back vulture (D. C. Houston & J. E. Cooper, personal communication). The only part of the gut anterior to the proventriculus is the crop and, as we shall see later, this has its own protective mechanism.

A common complication of feeding high levels of antibiotics to animals is a subsequent enteric infection resulting from the upgrowth of organisms which are not normally pathogenic. Implicit in this finding is the conclusion that the indigenous microflora of the gut normally protects the animal against some types of enteric disease. The nature of this protection differs in different parts of the intestine.

Balish & Phillips (1966*a*) found that normal chickens were more resistant to crop infections with *C. albicans* than were germ-free chickens. They suggested that *E. coli* might be responsible for this protective effect (Balish & Phillips, 1966*b*). More recently it has been shown that a lactobacillus occurs in close association with the epithelial lining of the chicken crop (Pl. 1C (Fuller & Turvey, 1971, Fuller & Brooker, unpublished)). This lactobacillus is firmly and specifically attached, has been found in all flocks examined, occurs throughout the life of the bird and is unaffected by drastic changes in diet. In short, it appears to be inextricably associated with the chicken, suggesting a possible symbiotic relationship. Lactobacilli which adhere to chicken-crop epithelial cells *in vitro* have also been isolated from the quail, pheasant, pigeon and Carolina wood duck. They were not found in the Bahama pintail, Canada goose, parrot or budgerigar or any of the 10 species of mammal examined (Fuller, 1973*a*).

When these lactobacilli are eliminated from the chicken crop by feeding high levels of penicillin there is an increase in the coliform population. Moreover, when *E. coli* is established as a monocontaminant in germ-free animals it reaches a population level higher than in conventional animals. If lactobacilli are introduced into the gut the pH of the crop content falls from 6·0 to 4·5 and the number of *E. coli* decreases. Bullen & Willis (1971) have shown that the increased resistance to gastro-enteritis which is characteristic of breast-fed babies was at least in part dependent on the presence of large numbers of lactobacilli in the gut and the maintenance of a low pH.

In the chicken and many other birds the oesophagus is expanded to form a crop where food is often held for several hours before being passed on into the acid-secreting part of the intestinal tract (proventriculus). Some mechanism is therefore needed in the crop to control the bacteria in the food until they can be dealt with in the proventriculus. It is suggested that adhesion of lactobacilli to the epithelium of the crop ensures a carry-over of a large inoculum so that by force of numbers the lactobacilli can exploit the food before other less desirable bacteria become established. In this way conditions inimical to the growth of bacteria generally are rapidly established. The potential use of this phenomenon in protection against enteric disease has been discussed by Fuller (1972).

Attempts by Fuller (1962) to detect antibacterial substances in the small intestine of chickens were only partially successful. Using *Cl.* (*welchii*) *perfingens* as the test organism and a method of assay which had proved successful in the pig (Fuller & Moore, 1967) he was not able to obtain consistent results. The antibacterial effect of bile *in vitro* is well known to all bacteriologists but its role *in vivo* is not well established. Floch, Gershengoren, Elliott and Spiro (1971) found that simple bile acids were more active than conjugated ones. Since it is claimed that bile acids of the chicken are taurine conjugates (Haslewood, 1967), their antibacterial role may be minimal unless adequate amounts of simple bile acids are released by bacterial degradation.

The rodent caecum contains a mechanism which is inhibitory to *Sal. typhimurium*. The basis of this protection has been studied by many workers and was recently reviewed by Hentges (1970). The inhibition is the result of a combination of high levels of volatile fatty acids, low pH and low oxidation–reduction potential. In the caecum of the chick Royal & Mortimer (1972) have shown that the growth of *Sal. typhimurium* is inhibited by bacteria. Their preliminary studies show that the anti-*Salmonella* factor(s) is heat stable and extractable with ether. They suggest that it may be similar to the volatile fatty-acid system of the rodent caecum.

Workers in Finland have recently shown that the number of chickens carrying *Salmonella* could be drastically reduced by dosing the birds soon after hatching with a suspension of gut content from adult chickens (Nurmi & Rantala, 1973). Thus it seems that the modern practice of rearing the chickens away from the hen and under conditions in which the opportunity to acquire a normal microflora is limited by the high standard of hygiene, may result in their increased susceptibility to infection.

The local intraluminal protection afforded by the flora of the gut is perhaps predictable, but what is surprising is the effect that gut commensals have on systemic immunity. Elimination of certain Gram-negative bacteria from the intestinal tract of mice, either by rearing the animals under clean (specified pathogen-free) conditions (Dubos & Schaedler, 1960) or by feeding antibacterial substances (Dineen, 1961), decreased their resistance to experimental infection. Similarly it was found that chickens mono-associated with *Cl. perfringens* had higher levels of serum bactericidins for *Sal. typhi* than did the germ-free controls (Hasegawa, 1959). Dineen (1961) suggested that this effect could be explained by the stimulation of non-specific defence mechanisms, and it has been shown that the digestive capacity of macrophages from germ-free mice is less than that of cells from conventionally reared animals (Bauer, Paronetto, Burns &

Einheber, 1966). The possibility that migration of bacteria from the gut is necessary for the systemic response is worth considering since it has already been shown that this is a common occurrence in young chicks (Fuller & Jayne-Williams, 1968) and it has been suggested that it might continue undetected throughout life (Fuller & Jayne-Williams, 1970*a*). Recently the bursa of Fabricius has been shown to harbour a large population of bacteria (Fuller, 1973*b*) which differs at least quantitatively from that found in the gut. Although the bursa does not itself produce antibody it might be an important site for antibody stimulation.

The similarity between the germ-free animals being contaminated experimentally and the sterile newly hatched chick acquiring its indigenous flora is inescapable. It would seem that the early colonization by the right organisms may be an important factor in the development of neonatal resistance.

IV. SUMMARY

1. The immunological response of the chicken continues to mature after hatching and it is suggested that both embryonic and neonatal chickens are at least partly dependent on non-specific mechanisms for protection against infection.

2. Two forms of non-specific antimicrobial defence can be identified in the egg: (*a*) a physical defence provided by the shell, shell membranes and albuminous sac, and (*b*) a chemical defence provided by the albumen and, possibly, the shell membranes.

3. The shell appears to be the major component in the physical defence system. Through being water-resistant and/or water-repellent, it impedes the flooding of the pores and thus the transfer of micro-organisms from the surface of the shell to the shell membranes. The latter appears to offer only a temporary impediment to the movement of bacteria. Bacteria which penetrate the shell membranes are prevented from making contact with the yolk of freshly laid eggs because of the barrier imposed by the albuminous sac.

4. There is little evidence to support the widely held belief that lysozyme is a major component in the chemical defence of eggs against infection. It is most likely that, through forming a network of fibres with ovomucin in the albuminous sac, lysozyme contributes to the physical defences of the egg.

5. A subtle form of chemical defence has evolved in the albumen. The white is inimical to micro-organisms yet non-toxic to the extra-embryonic membranes and the tissues of the embryo. Moreover, it is probable that it can protect the embryo from small numbers of bacteria and prevent also the autolysis of bacteria which could be expected to release pharmacologically active substances.

6. Ovotransferrin and the alkaline (pH 9·5) reaction of the albumen appear to be the principal components of the chemical defence. The first-mentioned prevents micro-organisms from acquiring the Fe^{3+} needed for their growth. The alkaline reaction is at the limits of tolerance of the majority of commonly occurring micro-organisms.

7. The wall of the chicken gut is not impermeable to bacteria. In the newly hatched chick the level of resistance is low, and intestinal bacteria which move across the gut wall can be detected in the liver and other tissues.

8. Compared with mammals the non-specific serum factors have been little studied in the chicken. Complement is present in 18-day-old embryos and interferon can be induced in embryos.

9. The body temperature of the neonatal chicken rises. This may accelerate clearance of bacteria by raising the rate of phagocytosis.

10. From hatching onwards the proventriculus and gizzard contain a level of hydrochloric acid which is lethal to many bacteria, including *Salmonella*.

11. In the crop lactobacilli are attached to the epithelium. These lactobacilli produce large amounts of lactic acid and the resulting pH of 4·5 is sufficiently low to prevent growth of other bacteria.

12. Cultures of caecal contents inhibit *Salmonella typhimurium* and it is suggested that the antibacterial system involved depends on volatile fatty acids for its activity.

13. The early establishment of a normal intestinal microflora appears to be an important factor in the resistance of chickens to *Salmonella* infections.

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VI. REFERENCES

ALDERTON, G., WARD, W. H. & FEVOLD, H. L. (1946). Identification of the bacteria-inhibiting, iron-binding protein of egg white as conalbumin. *Archs Biochem.* **11**, 9–13.

ALLS, A. A., BENTON, N. J., KRAUSS, W. C. & COVER, M. S. (1963). The mechanics of treating hatching eggs for disease prevention. *Avian Dis.* **7**, 89–97.

ALLS, A. A., COVER, M. S., BENTON, N. J. & KRAUSS, W. C. (1964). Treatment of hatching eggs for disease prevention – factors affecting permeability and a visual detection of drug absorption. *Avian Dis.* **8**, 245–56.

ALMQUIST, N. J. & HOLST, W. F. (1931). Variability of shell porosity in the hen's egg. *Hilgardia* **6**, 61–72.

VAN ALTEN, P. J. & SCHECHTMAN, A. M. (1963). Immunity in embryos. Anaphylaxis in young chicks suggests a new explanation for some puzzling properties of embryos. *J. exp. Zool.* **153**, 187–96.

ANDREWS, E. C. & MCKINNON, G. E. (1961). Studies on the resistance of fowl to pneumococcal infection. *Am. J. Path.* **39**, 579–88.

ATWAL, O. S. & MACFARLAND, L. Z. (1966). A morphologic and cytochemical study of erythrocytes of *Coturnix coturnix japonica*. *Am. J. vet. Res.* **27**, 1059–65.

AYRES, J. C. & TAYLOR, B. (1956). Effect of temperature on microbial proliferation in shell eggs. *Appl. Microbiol.* **4**, 356–9.

BALISH, E. & PHILLIPS, A. W. (1966*a*). Growth, morphogenesis and virulence of *Candida albicans* after oral inoculation in the germ-free and conventional chick. *J. Bact.* **91**, 1737–43.

BALISH, E. & PHILLIPS, A. W. (1966*b*). Growth and virulence of *Candida albicans* after oral incoulation in the chick with a monoflora of either *Escherichia coli* or *Streptococcus faecalis*. *J. Bact.* **91**, 1744–9.

BANG, F. B. (1943). The course of experimental infection of the chick embryo with the virus of equine encephalomyelitis. *J. exp. Med.* **77**, 337–44.

BANKOWSKI, R. A. & KALETA, E. F. (1972). Relations in interferon production and other known properties between Cal 11914 and TCND strains of Newcastle disease virus. *Avian Dis.* **16**, 481–91.

BARON, S. & ISAACS, A. (1961). Mechanism of recovery from viral infection in the chick embryo *Nature, Lond.* **191**, 97–8.

BATEMAN, R. G. & ROWLEY, D. (1969). The natural resistance of pigeons to Type III pneumococcus. *Aust. J. exp. Biol. med. Sci.* **47**, 733–45.

BAUER, H., PARONETTO, F., BURNS, W. A. & EINHEBER, A. (1966). The enhancing effect of the microbial flora on macrophage function and the immune response. *J. exp. Med.* **123**, 1013–24.

BEAN, K. C. & MACLAURY, D. W. (1959). The bacterial contamination of hatching eggs and methods for its control. *Poult. Sci.* **38**, 693–8.

BELL, A. E. (1949). Physiological factors associated with genetic resistance to fowl typhoid. *J. infect. Dis.* **85**, 154–69.

BELL, G. R., HOSKINS, G. E. & HODGKISS, W. (1971). Aspects of the characterisation, identification and ecology of the bacterial flora associated with the surface of stream incubating Pacific salmon (*Oncorhynchus*) eggs. *J. Fish. Res. Bd Can.* **28**, 1511–25.

BELLAIRS, R. & BOYD, A. (1969). Scanning electron microscopy of the shell membranes of the hen's egg. *Z. Zellforsch.* **96**, 237–49.

BELLAIRS, R., HARKNESS, W. & HARKNESS, E. D. (1963). The vitelline membrane of the hen's egg: a chemical and electron microscopic study. *J. Ultrastruct. Res.* **8**, 339–59.

BIGLAND, C. H. (1972). The tissue localisation of *Mycoplasma meleagridis* in turkey embryos. *Can. J. comp. Med.* **36**, 99–102.

BIGLAND, C. H. & PAPAS, G. (1953). Experiments in egg penetration by *Salmonella*. *Can. J. comp. Med.* **17**, 105–7.

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.* **27**, 350–64.

BOARD, R. G. (1969). The microbiology of the hen's egg. *Adv. appl. Microbiol.* **11**, 245–81.

BOARD, R. G. & AYRES, J. C. (1965). The influence of temperature on bacterial infection of the hen's egg. *Appl. Microbiol.* **13**, 358–64.

BOARD, R. G., AYRES, J. C., KRAFT, A. A. & FORSYTHE, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.* **43**, 584–95.

BOARD, R. G. & HALLS, N. A. (1973*a*). The cuticle: a barrier to water and particle uptake by the shell of the hen's egg. *Br. Poult. Sci.* **14**, 69–97.

BOARD, R. G. & HALLS, N. A. (1973*b*). Water uptake by the eggs of mallards and guinea fowl. *Br. Poult. Sci.* **14**, 311–14.

BOARD, R. G. & HALLS, N. A. (1973*c*). The effect of iron on the growth of *Escherichia coli* in albumen taken from the hen's egg. *Br. Poult. Sci.* **14**, 357–69.

BRANT, A. W. & STARR, P. B. (1962). Some physical factors related to egg spoilage. *Poult. Sci.* **41**, 1468–73.

BROOKS, J. (1960). Mechanisms of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.* **23**, 499–509.

BROOKS, J. & TAYLOR, D. I. (1955). Eggs and egg products. *Rep. Fd Invest. Bd* no. 60. London: H.M.S.O.

BROOKS, J. & HALE, H. P. (1959). The mechanical properties of the thick white of the hen's egg. *Biochim. biophys. Acta* **32**, 237–50.

BROOKS, J. & HALE, H. P. (1961). The mechanical properties of the thick white of the hen's egg. II. The relation between rigidity and composition. *Biochim. biophys. Acta* **46**, 289–301.

BRUNE, K., LEFFEL, M. S. & SPITZNAGEL, J. K. (1972). Microbicidal activity of peroxidaseless chicken heterophile leukocytes. *Infect. immunity* **5**, 283–7.

BÜCHLI, K. (1967). De invloed van het wassen op de microbiologische hondbaakheid van het ei. *Institut voor de Pluimveeteelt 'Het spelderholt' Beekbergen, Medeling* no. 145.

BUDDINGH, G. J. & POLK, A. D. (1939). The pathogenesis of meningococcus meningitis in the chick embryo. *J. exp. Med.* **70**, 499–510.

BULLEN, C. L. & WILLIS, A. T. (1971). Resistance of the breast fed infant to gastroenteritis. *Br. Med. J.* **3**, 338–43.

CARLSON, H. C., SWEENEY, P. R. & TOKARYK, J. M. (1968). Demonstration of phagocytic and trephocytic activities of chicken by microscopy and vital staining techniques. *Avian Dis.* **12**, 700–15.

CLARKE, R. M. & HARDY, R. N. (1970). Structural changes in the small intestine associated with the uptake of polyvinyl pyrrolidone by the young ferret, rabbit, guinea-pig and chicken. *J. Physiol.* **209**, 669–87.

COLLINS, F. M. (1967). Serum mediated killing of three group D salmonellae. *J. gen. Microbiol.* **46**, 247–53.

COOKE, A. S. & BALCH, D. A. (1970). Studies of membrane, mammillary cores and cuticle of the hen's egg shell. *Br. Poult. Sci.* **11**, 345–52.

COTTERILL, O. J. & WINTER, A. R. (1955). Egg white lysozyme: the effect of pH on the lysozyme–ovomucin interaction. *Poult. Sci.* **34**, 679–86.

COX, H. A., DAVIS, B. H., WATTS, A. D. & COLMER, A. R. (1972). *Salmonella* in the laying hen. 2. The effect of simulated digestive tract pH levels on the survival of three species of *Salmonella*. *Poult. Sci.* **51**, 1268–70.

CYBULSKA, J. & JELJASZEWICZ, J. (1966). Bacteriostatic activity of serum against staphylococci. *J. Bact.* **91**, 953–62.

DHINGRA, L. D., PARRISH, W. B. & VENZKA, W. G. (1969). Electron microscopy of granular leukocytes of chicken (*Gallus domesticus*). *Am. J. vet. Res.* **30**, 637–42.

DINEEN, P. (1961). The effect of alterations in intestinal flora on host resistance to systemic bacterial infection. *J. infect. Dis.* **109**, 280–6.
DRENT, R. H. (1967). *Functional Aspects of Incubation in the Herring Gull* (Larus argentatus *Pont.*). Ph.D. Thesis. Rijksuniversiteit te Groningen.
DRENT, R. H. (1972). Adaptive aspects of the physiology of incubation. *Proceedings of the XVI International Ornithological Congress*. Leiden: Brill.
DUBOS, R. J. & SCHAEDLER, R. W. (1960). The effect of the intestinal flora on the growth rate of mice and on their susceptibility to experimental infections. *J. exp. Med.* **111**, 407–17.
DUCAY, E. D., KLINE, L. & MANDELES, S. (1960). Free amino acid content of infertile chicken eggs. *Poult. Sci.* **39**, 831–5.
FARNER, D. S. (1942). The hydrogen ion concentration in avian digestive tracts. *Poult. Sci.* **21**, 445–50.
FERDINANDOV, V. V. (1944). Quoted from Zagaevsky & Lutikova (1944).
FLEMING, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc.* B **93**, 306–17.
FLEMING, A. & ALLINSON, V. D. (1924). On the antibacterial power of egg white. *Lancet*, **206**, 1303–7.
FLETCHER, J. (1971). The effect of iron and transferrin on the killing of *Escherichia coli* in fresh serum. *Immunology* **20**, 493–500.
FLOCH, M. H., GERSHENGOREN, W., ELLIOTT, S. & SPIRO, H. M. (1971). Bile acid inhibition of the intestinal microflora – a function for simple bile acids? *Gastroenterology* **61**, 228–33.
FROMM, D. & MONROE, R. J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg shell permeability. *Fd Technol., Champaign* **14**, 401–3.
FUJII, S. & TAMURA, T. (1970). Scanning electron microscopy of shell formation in hen's eggs. *J. Fac. Fish. Anim. Husb. Hiroshima Univ.* **9**, 65–81.
FULLER, R. (1962). *Studies on the gut flora of the pig and chick*. Ph.D. Thesis, University of Reading.
FULLER, R. (1972). Bacteria that stick in the gut. *New Scient.* **56**, 506–7.
FULLER, R. (1973*a*). Ecological studies on the lactobacillus flora associated with the crop epithelium of the fowl. *J. appl. Bact.* **36**, 131–9.
FULLER, R. (1973*b*). Differences in the microfloras of the intestine and bursa of Fabricius as illustrated by the coliform/lactobacillus ratio in the two sites. *Br. Poult. Sci.* **14**, 221–4.
FULLER, R. & JAYNE-WILLIAMS, D. J. (1968). The origin of bacteria recovered from the peritoneum and yolk sac of healthy chickens. *Br. Poult. Sci.* **9**, 159–63.
FULLER, R. & JAYNE-WILLIAMS, D. J. (1970*a*). Resistance of the fowl (*Gallus domesticus*) to invasion by its intestinal flora. II. Clearance of translocated intestinal bacteria. *Res. Vet. Sci.* **11**, 368–74.
FULLER, R. & JAYNE-WILLIAMS, D. J. (1970*b*). Resistance of the fowl (*Gallus domesticus*) to invasion by its intestinal flora. I. The effect of hormonal bursectomy on the invasiveness of the intestinal microflora of the fowl. *Res. vet. Sci.* **11**, 363–7.
FULLER, R. & MOORE, J. H. (1967). The inhibition of the growth of *Clostridium welchii* by lipids isolated from the contents of the small intestine of the pig. *J. gen. Microbiol.* **46**, 23–41.
FULLER, R. & TURVEY, A. (1971). Bacteria associated with the intestinal wall of the fowl (*Gallus domesticus*). *J. appl. Bact.* **34**, 617–22.
GABAYEVA, N. S. (1962). Antibiotic properties of the jelly of frogs' eggs. *Vest. leningr. U-Ta Adsk. Univ.* **15**, 25–32.
GARIBALDI, J. A. (1960). Factors in egg white which control growth of bacteria. *Fd Res.* **25**, 337–44.
GARIBALDI, J. A. (1970). Role of microbial iron transport compounds in the bacterial spoilage of eggs. *Appl. Microbiol.* **20**, 558–60.
GARIBALDI, J. A. & BAYNE, H. G. (1960). Iron and bacterial spoilage of shell eggs. *J. Fd Sci.* **27**, 57–9.
GILLESPIE, J. M. & SCOTT, W. J. (1950). Studies in the preservation of shell eggs. IV. Experiments in the mode of infection by bacteria. *Aust. J. appl. Sci.* **1**, 514–31.
GIRFANOVA, H. N. (1949*a*). Prosticidal activity of albumen of egg. *Dokl. Akad. Nauk SSSR* **68**, 941–3.
GIRFANOVA, H. N. (1949*b*). Changes in the bactericidal properties of natural albumen and lysozyme of the chicken egg during incubation. *Dokl. Akad. Nauk SSSR* **68**, 1105–8.
GOODPASTURE, E. W. & ANDERSON, K. (1937). The problem of infection as presented by bacterial invasion of the chorio-allantoic membrane of chick embryo. *Am. J. Path.* **13**, 149–74.
GORDON, R. F. & TUCKER, J. F. (1954). Behaviour of *Pseudomonas* sp. and the natural occurrence of the organism in the fowl and its environment. Contribution 109 of *10th World's Poult. Congress*, Edin. Dept. Agric. Scotland.
GREENFIELD, J. & BIGLAND, C. H. (1971). Detection of lysozyme in developing avian embryos. *Poult. Sci.* **50**, 1748–53.
HAINES, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of a new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.* **38**, 338–55.
HAINES, R. B. (1939). Microbiology in the preservation of the hen's egg. *Rep. Fd Invest. Bd* no. 47. London: H.M.S.O.

HAINES, R. B. & MORAN, T. (1940). Porosity of, and bacterial invasion through the shell of the hen's egg. *J. Hyg., Camb.* **40**, 453–61.

HAMDY, M. K., BROWN, W. E. & MCCARTHY, P. M. (1963). Epidemiological studies on bruised tissue. I. Source and portal of entry of microorganisms found in bruised tissue. *Bact. Proc.* p. 16.

HARRY, E. G. (1957). The effect on embryonic and chick mortality of yolk contamination with bacteria from the hen. *Vet. Rec.* **69**, 1433–9.

HARRY, E. G. (1963*a*). Some observations on the bacterial content of the ovary and oviduct of the fowl. *Br. Poult. Sci.* **4**, 63–70.

HARRY, E. G. (1963*b*). The relationship between egg spoilage and the environment of the egg when laid. *Br. Poult. Sci.* **4**, 91–100.

HARTUNG, T. E. & STADELMAN, W. J. (1963). *Pseudomonas fluorescens* penetration of egg shell membranes as influenced by shell porosity, age of egg and degree of bacterial challenge. *Poult. Sci.* **42**, 147–50.

HASEGAWA, H. (1959). Physiological significance of *Cl. welchii*: a study on anaerobic bacteria in the intestines of animals. *J. Chiba Med. Soc.* **34**, 1470–81.

HASLEWOOD, G. A. D. (1967). *Bile Salts*. London: Methuen.

HAWTHORNE, J. R. (1950). The action of egg white lysozyme on ovomucoid and ovomucin. *Biochim. biophys. Acta* **6**, 28–35.

HENTGES, D. J. (1970). Enteric pathogen–normal flora interaction. *Am. J. Clin. Nutr.* **23**, 1451–6.

HERPOL, C. & VAN GREMBERGEN, G. (1961). Le pH dans le tube digestif des oiseaux. *Annls Biol. anim. Biochim. Biophys.* **1**, 317–21.

HIRSCH, J. G. (1962). Cinemicrophotographic observations on granule lysis in polymorphonuclear leucocytes during phagocytosis. *J. exp. Med.* **116**, 827–33.

HIRSCH, J. G. (1972). The phagocytic defence system. *Symp. Soc. gen. Microbiol.* **22**, 59–74.

ISAACS, A. & LINDENMANN, J. (1957). Virus interference. I. The interferon. *Proc. R. Soc.* B **147**, 258–67.

KARTHIGASU, K. & JENKIN, C. R. (1963). The functional development of the reticulo-endothelial system of the chick embryo. *Immunology* **6**, 255–63.

KARTHIGASU, K., JENKIN, C. R. & TURNER, K. J. (1964). The nature of the opsonins in adult hen serum and developing chick embryos to certain Gram-negative bacteria. *Aust. J. exp. Biol. med. Sci.* **42**, 499–510.

KARTHIGASU, K., READE, P. C. & JENKIN, C. R. (1965). The functional development of the reticulo-endothelial system. III. The bactericidal capacity of fixed macrophages of foetal and neonatal chicks and rats. *Immunology* **9**, 67–73.

KENT, R. (1961). The development of the phagocytic activity of the reticuloendothelial system in the chick. *J. Embryol. exp. Morph.* **9**, 128–37.

KOROTKOVA, G. P. (1957*a*). Antibiotic properties of the albuminous membrane of hen's egg. *Zh. Obshch. Biol.* **18**, 275–87.

KOROTKOVA, G. P. (1957*b*). Immunity of embryos and antibiotic properties of albumen membrane. *Zhatnse Obshch, Biologii* **17**, 275–85.

KOROTKOVA, G. P. (1959). The influence of antibiotic properties of albumen membranes on chicken pathogens. *Vest. leningr. U-Ta ser. biol.* **3**, 76–82.

KRAMER, T. T. & CHO, N. C. (1970). Transfer of immunoglobulins and antibodies in the hen's egg. *Immunology* **19**, 157–67.

LACK, D. (1968). *Ecological Adaptations for Breeding in Birds*. London: Methuen.

LANDAUER, W. (1967). The hatchability of chicken eggs as influenced by environment and heredity. *Storrs Agricultural Exp. Stn Monograph* **1**.

LASCHTSCHENKO, P. (1909). Uber die Keimtotende und entwicklungshemmende Wirkung huhnere Weiss. *Z. Hyg. InfektKrankh.* **64**, 419–27.

LILLIE, F. R. (1952). *The Development of the Chick*. New York: Henry Holt.

LONG, C. (1968). *Biochemists' Handbook*. London: Spon.

LORAH, M. E., FUNK, E. M. & FORWARD, J. (1954). Reducing spoilage in shell eggs by the use of fungicides. *Univ. Missouri Agric. Exp. Stn* 573.

LORENZ, F. W., STARR, P. B., STARR, M. P. & OGASAWARA, F. X. (1952). The development of *Pseudomonas* spoilage in eggs. 1. Penetration through the shell. *Fd Res.* **17**, 351–60.

MCCLAIN, M. E. & MODIN, S. H. (1955). Experimental studies on vesicular stomatitis virus. I. Factors influencing infectivity titrations in the chick embryo. *J. infect. Dis.* **97**, 280–6.

MALKINSON, M. (1965). The transmission of passive immunity to *Escherichia coli* from mother to young in the domestic fowl (*Gallus domesticus*). *Immunology* **9**, 311–17.

MARSHALL, M. E. & DEUTSCH, H. F. (1950). Some protein changes in fluids of the developing chicken embryo. *J. biol. Chem.* **185**, 155–61.

MERKENSCHLAGER, M., LOSCH, U., KIRCHNER, B., RIEDEL, G. & GRUBER, F. (1967). Immunoglobulingehalte im Serum junger, gnotobiotischer Mastkuken. *Z. Tierphysiol. Tierernahr. Futtermittelk.* **22**. 337–49.

METCHNIKOFF, E. (1907). *The Prolongation of Life.* London: Heinemann.
MILLER, W. A. & CRAWFORD, L. B. (1953). Some factors influencing bacterial populations of eggs. *Poult. Sci.* **32**, 303–4.
MIZEJEWSKI, G. J. & RAMM, G. M. (1969). Phagocytosis as related to the reticulo-endothelial system (RES) in the developing chick. *Growth* **33**, 47–56.
MORAHAN, P. S. & GROSSBERG, S. E. (1970*a*). Age related cellular resistance of the chicken embryo to viral infections. I. Interferon and natural resistance to myxoviruses and vesicular stomatitis virus. *J. infect. Dis.* **121**, 615–23.
MORAHAN, P. S. & GROSSBERG, S. E. (1970*b*). Age related cellular resistance of the chicken embryo to viral infections. II. Inducible resistance produced by influenza virus and *Escherichia coli*. *J. infect. Dis.* **121**, 624–33.
MORAN, T. & HALE, H. P. (1936). Physics of the hen's egg. I. Membranes in the egg. *J. exp. Biol.* **13**, 35–41.
MORGAN, G. W. & GLICK, B. (1972). A quantitative study of serum proteins in bursectomised and irradiated chickens. *Poult. Sci.* **51**, 771–8.
MOUCHAN, N. A. (1964). Antimicrobial properties of the albumen membrane of chicken, duck and guinea fowl. *Vestnik. leningr. Univ. ser. biol.* **3**, 54–65.
NEW, D. A. T. (1959). The adhesive properties and expansion of the chick blastoderm. *J. Embryol. exp. Morph.* **7**, 146–64.
NICOL, T., COX, F. E. G., BILBEY, D. L. J. & STRACHEN, J. E. (1962). Development of reticulo-endothelial system in avian embryos. *Nature, Lond.* **194**, 105–6.
NURMI, E. & RANTALA, M. (1973). New aspects of *Salmonella* infection in broiler production. *Nature, Lond.* **241**, 210–11.
OREL, V. (1959). The *Pseudomonas* spoilage of eggs laid by individual hens. *Poult. Sci.* **38**, 8–12.
OSUOJI, C. I. (1971). Acid glycosaminoglycan of egg shell membranes. *Biochim. biophys. Acta* **244**, 481–3.
PAYNE, L. N. (1968). Eggs in virology. In *Egg Quality: a study of the hen's egg* (ed. T. C. Carter). Edinburgh: Oliver & Boyd.
PHELPS, R. A., SHENSTONE, F. S., KEMMERER, A. R. & EVANS, R. J. (1965). A review of cycloprenoid compounds: biological effects of some derivatives. *Poult. Sci.* **44**, 358–94.
POTTER, E. V. & STOLLERMAN, G. H. (1961). The opsonization of bentonite particles by γ-globulin. *J. Immunol.* **87**, 110–18.
PRAGER, F. M., ARNHEIM, N., MROSS, G. A. & WILSON, A. C. (1972). Amino acid sequence studies on bobwhite quail egg white lysozyme. *J. biol. Chem.* **247**, 2905–16.
PRINCE, W. R. (1967). Evidence for lysozyme depletion in Rhode Island Red chicken exposed to fowl typhoid. *Poult. Sci.* **46**, 1308.
PRINCE, W. R. (1971). The *in vitro* inhibition of *Salmonella gallinarum* by pancreatic-intestinal extracts from chicken exposed to fowl typhoid. *Poult. Sci.* **50**, 1069–71.
PRINCE, W. R. & GARREN, H. W. (1966). An investigation of the resistance of White Leghorn chickens to *Salmonella gallinarum*. *Poult. Sci.* **45**, 1149–53.
PRUNESCO, P. & PRUNESCO, C. (1972). Phagocytosis by avian red cells. *Naturwissenschaften* **59**, 41.
RAMACHANDRAN, S. P. & FRASER, G. (1971). Studies on the virus of Aujeszky's disease. II. Pathogenicity for chicks. *J. comp. Pathol.* **81**, 55–62.
RAUCH, W. (1952). The influence of egg-shell porosity on the number of chickens hatched from incubating eggs. *Poult. Sci.* **31**, 589–94.
RIZK, S. S., AYRES, J. C. & KRAFT, A. A. (1966). Effect of holding conditions on the development of *Salmonella* in artificially inoculated hen's eggs. *Poult. Sci.* **45**, 825–9.
ROBINSON, D. S. & MONSEY, J. B. (1969*a*). A quantitative study of a reduced ovomucin–natural lysozyme interaction. *Biochem. J.* **115**, 64P.
ROBINSON, D. S. & MONSEY, J. B. (1969*b*). A reduced ovomucin–reduced lysozyme complex from egg white. *Biochem. J.* **115**, 64P.
ROBINSON, D. S. & MONSEY, J. B. (1971). Studies on the composition of egg white ovomucin. *Biochem. J.* **121**, 537–47.
ROBINSON, D. S. & MONSEY, J. B. (1972). Changes in the composition of ovomucin during liquefaction of thick egg white. *J. Sci. Fd Agric.* **23**, 29–38.
ROGERS, H. J. (1967). Bacteriostatic effects of horse sera and serum fractions on *Clostridium welchii* type A and the abolition of bacteriostasis by iron salts. *Immunology* **12**, 285–302.
ROGERS, H. J. & PERKINS, H. R. (1968). *Cell Walls and Membranes.* London: Spon.
RON, E. Z. & DAVIS, B. D. (1971). Growth rate of *Escherichia coli* at elevated temperatures: limitation by methionine. *J. Bact.* **107**, 391–6.
ROSE, M. E. & ORLANS, E. (1962). Fowl antibody. III. Its haemolytic activity with complements of various species and some properties of fowl complement. *Immunology* **5**, 633–41.

ROSSER, F. T. (1942). Preservation of eggs. II. Surface contamination on egg-shell in relation to spoilage. *Can. J. Res.* **20D**, 291–6.

ROYAL, W. A. & MORTIMER, M. D. (1972). Inhibition of *Salmonella typhimurium* by fowl caecal cultures. *Res. vet. Sci.* **13**, 184–5.

SCHADE, A. L. & CAROLINE, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae, Staphylococcus aureus, Escherichia coli* and *Saccharomyces cerevisiae*. *Science, N.Y.* **100**, 14–15.

SCHOLES, J. C. & HUTT, F. B. (1942). The relationship between body temperature and genetic resistance to *S. pullorum* in the fowl. *Cornell Univ. Agric. exp. Sta. Memoir* 244.

SETO, F. (1972). Effects of temperature on antibody production by chicken immunocytes. *Poult. Sci.* **51**, 1222–8.

SEVIOUR, E. M. & BOARD, R. G. (1972). Bacterial growth in albumen taken from the eggs of domestic hens and waterfowl. *Br. Poult. Sci.* **13**, 557–75.

SEVIOUR, E. M., SYKES, F. R. & BOARD, R. G. (1972). A microbiological survey of the incubated eggs of chickens and waterfowl. *Br. Poult. Sci.* **13**, 549–56.

SHARP, B. F. & STEWART, G. F. (1936). Effect of relative humidity on the growth of moulds on eggs in storage. *Mem. Cornell Univ. agric. Exp. Stn* 91.

SHARP, P. F. & WHITAKER, R. (1927). The relation of the hydrogen ion concentration of egg white to its germicidal action. *J. Bact.* **14**, 17–46.

SIBLEY, C. G. (1960). The electrophoretic patterns of avian egg white proteins as taxonomic characters. *Condor* **59**, 166–91.

SIBLEY, C. G. (1970). A comparative study of the egg white proteins of passerine birds. *Bulletin no.* **32**, *Peabody Museum of Natural History, Yale University.*

SMITH, A. J. H. (1934). The relations between yolk and white in the hen's egg. *J. exp. Biol.* **11**, 228–42.

SMITH, H., KEPPIE, J., PEARCE, J. H., FULLER, R. & WILLIAMS, A. E. (1961). The chemical basis of the virulence of *Brucella abortus*. I. Isolation of *Br. abortus* from bovine foetal tissue. *Brit. J. exp. Path.* **42**, 631–7.

SMITH, H., WILLIAMS, A. E., PEARCE, J. H., KEPPIE, J., HARRIS-SMITH, P. W., FITZ-GEORGE, R. B. & WITT, K. (1962). Foetal erythritol: a cause of localisation of *Brucella abortus* in bovine contagious abortion. *Nature, Lond.* **193**, 47–49.

SMOLELIS, A. N. & HARTSELL, S. E. (1951). Occurrence of lysozyme in bird egg albumins. *Proc. Soc. exp. Biol. Med., N.Y.* **76**, 455–6.

SOLOMON, J. B. (1964). The onset of immunological competence in the chicken. *Folia biol. Praha* **10**, 268–73.

SOLOMON, J. B. (1966). Induction of antibody formation to goat erythrocytes in the developing chick embryo and effects of maternal antibody. *Immunology* **11**, 89–96.

SOLOMON, J. B. (1968*a*). Immunity to *Salmonella gallinarum* during ontogeny of the chicken. I. Onset of resistance to infection; the minor role of opsonins. *Immunology* **15**, 197–206.

SOLOMON, J. B. (1968*b*). Immunity to *Salmonella gallinarum* during ontogeny of the chicken. III. Bactericidal antibody. *Immunology* **15**, 219–26.

SOLOMON, J. B. (1971). *Foetal and Neonatal Immunity*. North-Holland Publishing Co.: Amsterdam and London.

STOKES, J. L., OSBORNE, W. W. & BAYNE, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Fd Res.* **21**, 510–18.

STUART, L. S. & MCNALLY, E. H. (1943). Bacteriological studies on the egg shell. *U.S. Egg Poult. Mag.* **49**, 28–47.

THEODORE, T. S. & SCHADE, A. L. (1965*a*). Growth of *Staphylococcus aureus* in media of restricted and unrestricted inorganic iron availability. *J. gen. Microbiol.* **39**, 75–83.

THEODORE, T. S. & SCHADE, A. L. (1965*b*). Carbohydrate metabolism of iron-rich and iron-poor *Staphylococcus aureus*. *J. gen. Microbiol.* **40**, 385–96.

TOIVANEN, P., TOIVANEN, A. & GOOD, R. A. (1972). Ontogeny of bursal function in chicken. III. Immunocompetent cell for humoral immunity. *J. exp. Med.* **136**, 816–31.

TOKIN, B. P. (1964). Ecology of embryonic development. Immunity of embryos. *Folia biol. Praha* **10**, 61–70.

TONER, P. G. (1965). Development of the acid secretory potential in the chick embryo proventriculus. *J. Anat.* **99**, 389–98.

TOPP, R. C. & CARLSON, H. C. (1972). Studies on avian heterophils. II. Histochemistry. *Avian Dis.* **16**, 369–73.

TOSHKOV, A. & SHIROVA, L. (1970). Le proprieta antibatteriche dell'albume d'novo come fattore protettivo por l'embrione di pollo. *Estratto dalla Rivista* **25**, 842–6.

TRUST, T. J. (1972). The bacterial population in vertical flow tray hatcheries during incubation of salmonid eggs. *J. Fish. Res. Bd Can.* **29**, 567–71.

TUNG, M. A. & RICHARDS, J. F. (1972). Ultrastructure of the hen's egg shell membranes by electron microscopy. *J. Fd Sci.* **37**, 277–81.

TYLER, C. (1955). Studies on egg shells. VI. The distribution of pores in egg shells. *J. Sci. Fd Agric.* **3**, 170–6.

TYLER, C. (1969*a*). The distribution of pores in the egg shells of the domestic fowl: a further study. *Br. Poult. Sci.* **10**, 357–80.

TYLER, C. (1969*b*). Avian eggs: their structure and characteristics. *Int. Rev. gen. exp. Zool.* **4**, 82–127.

UHLENBRUCK, G., SPRINGER, I., HERMAN, G. & FRANKE, H. (1972). Fish eggs: another new source of polyvalent proteinase-isoinhibitor. *Z. Naturf.* **27***b*, 322–3.

VADEHRA, D. V., BAKER, R. C. & NAYLOR, N. B. (1969). *Salmonella* infection of cracked eggs. *Poult. Sci.* **48**, 954–7.

VADEHRA, D. V., BAKER, R. C. & NAYLOR, N. B. (1970*a*). Role of cuticle in spoilage of chicken eggs. *J. Fd Sci.* **35**, 5–6.

VADEHRA, D. V., BAKER, R. C. & NAYLOR, N. B. (1970*b*). Infection routes of bacteria in chicken eggs. *J. Fd Sci.* **35**, 61–2

VOLKERT, M. & LARSON, J. H. (1965). Studies on immunological tolerance to LCM virus. 5. The induction of tolerance to the virus. *Acta path. microbiol. Scand.* **63**, 161–71.

VOS, J. C. (1964). Lysozyme lysis of Gram-negative bacteria without production of sphaeroplasts. *J. gen. Microbiol.* **35**, 313–7.

WARNER, N. L., SZENBERG, A. & BURNET, F. M. (1962). The immunological role of different lymphoid organs in the chicken. 1. Dissociation of immunological responsiveness. *Aust. J. exp. Biol. Med. Sci.* **40**, 373–88.

WARREN, L. G. & BORSOS, T. (1959). Studies on immune factors occurring in sera of chickens against the crithidia stage of *Trypanosoma cruzi*. *J. Immun.* **82**, 585–90.

WESTON, W. A. R. D. & HALNAN, E. T. (1928). 'Black spot' of eggs. *Poult. Sci.* **6**, 251–8.

WILLIAMS, J. (1962). Comparison of conalbumin and transferrin in the domestic fowl. *Biochem. J.* **83**, 355–64.

WILLIAMS, J. E. & WHITTEMORE, A. D. (1967). A method for studying microbial penetration through the outer structures of the avian egg. *Avian Dis.* **11**, 467–90.

WILLIAMS, J. E., DILLARD, L. H. & HALL, G. O. (1968). The penetration patterns of *Salmonella typhimurium* through the outer structure of chicken eggs. *Avian Dis.* **12**, 445–66.

WITSCHI, E. (1956). *Development of Vertebrates*. Philadelphia: Saunders.

WOLKEN, J. J. (1951). Structure of hen's egg membranes. *Anat. Rec.* **111**, 79–89.

YAMOMOTO, R. & BIGLAND, C. H. (1966). Infectivity of *Mycoplasma meleagridis* for turkey embryos. *Am. J. Vet. Res.* **27**, 326–30.

ZAGAEVSKY, J. S. & LUTIKOVA, P. O. (1944). Sanitary measure in egg breaking plants. *U.S. Egg. Poult. Mag.* **50**, 17–123.

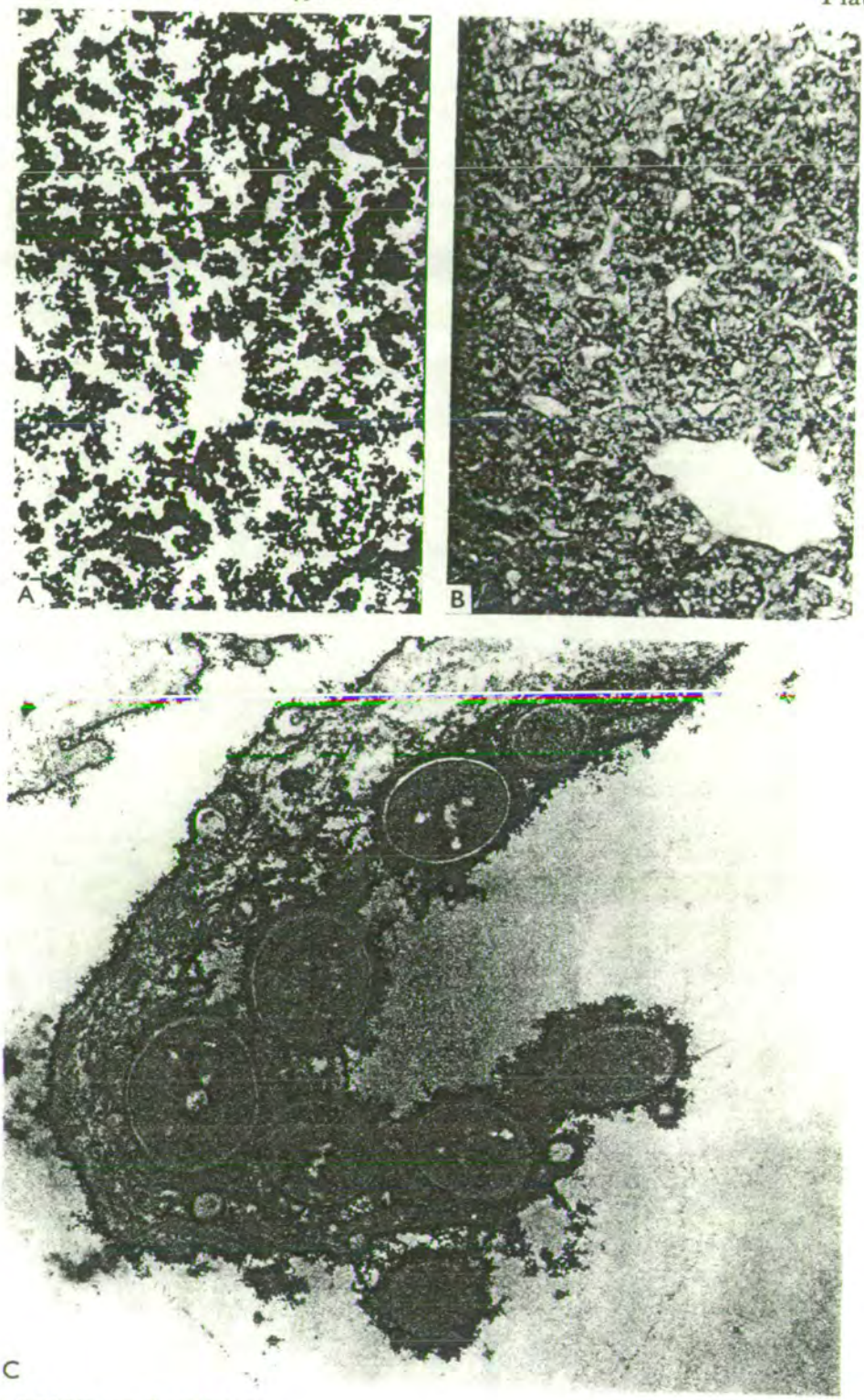
EXPLANATION OF PLATE

PLATE 1

A. Section through liver of newly hatched chicken to show lipid stores. Sudan Black, × 100. (A. Turvey & R. Fuller, unpublished.)

B. Similar section from week-old chicken.

C. Electron micrograph of thin section through wall of chicken crop showing lactobacilli attached to the epithelium. The darkly stained material surrounding the bacteria is carbohydrate and probably responsible for the adhesion of the bacteria to the epithelial cells. Alcian blue–lanthanum nitrate, × 28,800. (R. Fuller & B. E. Brooker, unpublished.)



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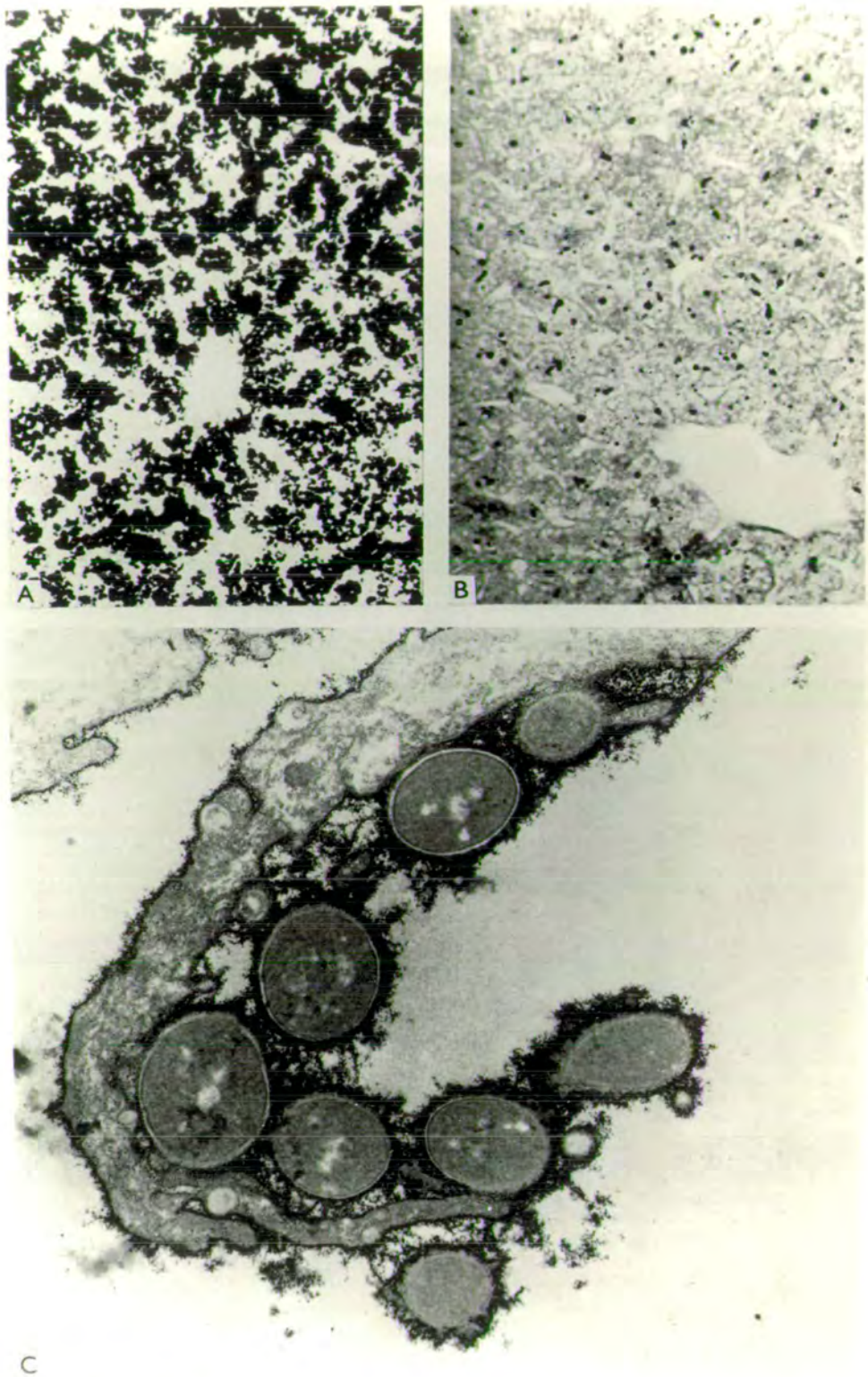
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The Avian Eggshell—a Resistance Network

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Contents

1. Introduction

MANY OF THE problems encountered in the day-to-day handling of the eggs of commerce can be attributed to a poor understanding of the biological roles that the avian eggshell has to perform. Persons concerned with table eggs seem intent on offering the housewife a clean shell; they commonly achieve this by washing eggs. It has been demonstrated repeatedly that if such an operation is poorly controlled there can be a high incidence of rotting in the washed eggs resulting from the flooding of the pore canals with contaminated water (Haines & Moran 1941).

Veterinarians who seek to break an infection route of mycoplasmas or salmonellas have focused attention on the egg because it offers an opportunity of producing specific pathogen-free progeny from stock that harbours a pathogen. Although the routine use of gnotobiotic chickens in research shows that shell sterilization can be achieved without harm to the embryo, problems are encountered when there is a move from the treatment of the relatively few eggs needed for germ-free chick production to the large number required for a breeding programme. Indeed the scale of the latter operation, even though freedom from a specific pathogen rather than from all microbes is the objective, may be such that the work cannot be done under conditions or with personnel deemed essential by the laboratory-based scientist. Although such difficulties can be identified easily, they may well prove to be subordinate to that of translocating an adequate amount of an antimicrobial agent across the shell of every egg in a batch. Failure to achieve this may well be due in large part to a poor appreciation of the form and function of the pore canal.

Over the past few years I have been made aware of the problems that can be encountered by those who seek to maintain, often by means of artificial incubation, a diverse range of bird species in bird gardens, etc. When an unacceptable incidence of rotting occurs in incubating eggs, there is a tendency to adopt control measures that have been evolved to counter such problems in the commercial incubation of the eggs of domestic hens, ducks and geese. Recent studies of eggshell structure (Tullett *et al.* 1976; Board *et al.* 1977; Board & Perrott 1979*a*,*b*; Board *et al.* 1980) have shown that

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there is a marked diversity in the morphology of the pore canal and especially in the form and chemical composition of the materials in or covering the outer orifice of the canal. This diversity was used to create an arbitrary classification (Board *et al.* 1977); subsequent studies have led to the revised classification given in Fig. 1. As nearly all the

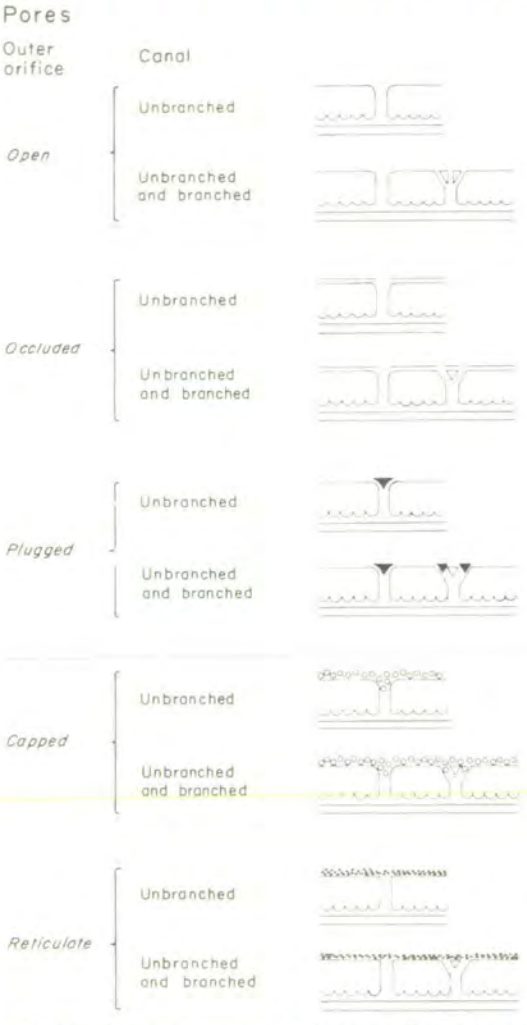

Fig. 1. An arbitrary classification of the pore systems in avian eggshells. It is based on the scheme of Board *et al.* (1977), the modifications resulting from the studies of Board & Perrott (1979*b*) and Board *et al.* (1980). Subcategories of some of the major divisions are A. Plugged pores—the plug may be organic as in the tinamou eggshell (Board & Perrott 1979*b*) or probably inorganic as in the rhea eggshell (Board & Perrott, unpublished observations), and B. Capped pores—three subcategories based on the chemical composition of the capping material can be defined, capping material: (*i*) organic, *e.g.* domestic hen; (*ii*) inorganic in crystalline form, *e.g.* some non-parasitic cuckoos have vaterite as a capping material (Board & Perrott 1979*a*), and (*iii*) inorganic in amorphous form, *e.g.* the Great Crested Grebe (Board *et al.* 1980). Examples of birds laying eggs of the types shown in the figure are: *open*, unbranched canal, pigeons and doves, branched canal, ostrich; *occluded*, unbranched canal, pheasant; *plugged*, unbranched canal, tinamou, branched canal, rhea; *capped*, unbranched canal, domestic hen, turkey, guinea fowl, duck and goose, branched canal, swans, and *reticulate*, unbranched canal, storks and branched, emu.

work on 'sanitation' of shells has been done with the eggs of the domestic hen, the results may be applicable to only one subcategory of the amended classification, namely pores capped with organic material. This type of pore is illustrated in Plate 1. Within the category of capped pores, there are in addition to those pores having organic capping material others on which the material is inorganic, either in a crystalline—commonly vaterite—(Tullett *et al.* 1976; Board & Perrott 1979*a*), or amorphous form (Board *et al.* 1980). With the last mentioned, the calcium may occur as the phosphate rather than the carbonate salt. Thus even within one category of the classification given in Fig. 1, diversity of chemical form could well impose problems in the selection of appropriate disinfectants because of the possibility of inactivation by the covering material. When the whole range of pore structures is considered in the context of simply washing the eggs without initiating rotting, there are as yet no guidelines to aid us.

The purpose of this article is to discuss the concept that an avian eggshell can be considered as a resistance network. Such a concept should aid those who set out to establish guidelines for the washing and disinfection of hatching eggs. Perhaps its major contribution will be to those studies concerned with dosing eggs with antimicrobial agents so that an infection route is broken.

2. The Functions of the Eggshell

The eggshell makes many contributions to the well-being of the embryo (Table 1). This article will consider only those associated with the pores.

A. Pore function

Although the existence and functions of pores have been known for upwards of two centuries, it is only in the past decade that observations have been sufficiently detailed to permit an interpretation of what appears to be opposing functions: the exchange of respiratory gases and the conservation of water present in the egg at oviposition.

As the oxygen molecule is larger than that of water and as the exchange of respiratory gases across the shell is dependant upon diffusion, the pores provide portals not only for the movement of gases but also the outward movement of water vapour. The pores may therefore be considered to be functionally analogous to stomata. There is, however, one major difference. The size of the stomatal orifice can be regulated to

TABLE 1

Contributions of avian eggshell to the well-being of the embryo

Exchange of respiratory gases
Prevention of asphyxiation
Antimicrobial defence
Mechanical protection
Reservoir of Ca^{2+}
Camouflage

accommodate changes in the water status of the plant so that damage due to excessive water loss is avoided. As the pores in the eggshell are nothing more than tubes in calcite, there is apparently no mechanism that can control the rate of diffusion apart from the steepness of the diffusion gradient obtaining across the shell. As the water activity of the shell membranes is *ca.* 0·98, the RH of the atmosphere surrounding the egg would have to be 98% to prevent water loss from the egg. The observation (Board *et al.* 1979) that a strain of pseudomonad can grow on and digest the glycoprotein cuticle on pieces of hens' eggshell suspended in an atmosphere equilibriated at RH 98 suggests that eggshells having organic covers are poorly adapted to wet/humid conditions. Indeed there are many reports of mould growth on stored hens' eggs and in all cases growth was attributed to a high RH of the storage atmosphere (Board *et al.* 1979).

A perspective was given to the question of water loss from incubating eggs by Rahn and his collaborators (1976). From the results obtained with the eggs of several species of birds, they derived the formula:

$$Ap/L \cdot \Delta P_{H_2O} \cdot I \cdot 2{\cdot}24 \times 10^{-3} = F.W.$$

This takes account of the pore geometry (area Ap, length L) and the diffusion gradient (ΔP_{H_2O}). The practical outcome of selective pressures would appear to be shells endowed with pores sufficient in number and area to permit an optimal water loss (F) during incubation (I). Loss is associated with a progressive enlargement of the aircell such that immediately before hatching the embryo has a reservoir of O_2 to support lung breathing and space to move during the chipping of the shell leading to hatching. Thus it could be argued that selective pressures have favoured shells having a porosity sufficient for a particular amount of water to be lost from an egg and that the embryo's requirements for the exchange of respiratory gases have been a secondary trait in selection. From a microbiological standpoint, this information indicates that under conditions conducive to incubation an egg having an organic cover would be unlikely to be surrounded with an atmosphere containing sufficient water vapour for the growth of the cuticle-digesting pseudomonad isolated by Board *et al.* (1979).

B. Pore flooding

Needham (1942) concluded that independence of a need for an exogenous source of water was the last step in the evolution of cleidoic (closed box) eggs such as those of birds. Through achieving this state, the bird's egg may have become vulnerable to water. Thus the pores needed for 'chemical communication'—the exchange of respiratory gases—between the embryo and its environment have dimensions that could in theory support the capillary movement of water. If water were to be drawn into the pores, the embryo would be asphyxiated or infected (Board & Fuller 1974). The work of Haines & Moran (1941) can be taken as a milestone in our understanding of the factors involved in the initiation of rotting of stored hens' eggs.They recorded a low incidence of rots in eggs that had been immersed briefly in a bacterial suspension of the same temperature but a high incidence when the eggs were warmer than the suspension. They deduced that a small negative pressure was generated in the latter case because the contraction of the yolk and white was greater than that of the shell. This

caused contaminated water to be sucked into the pore canal. Their deduction has met with general acceptance even though experimental verification is still awaited.

Studies of factors contributing to the flooding of pore canals (Board & Halls 1973*a*,*b*) and the fine structure of pores (Board 1974, 1975; Tullett *et al.* 1975) led to the concept that avian eggshells are water-repellent or water-resistant. Work has therefore to be done to force water into the pores and the contraction of a warm egg in cold water is one way of achieving this end. In recent years a variety of other techniques have been developed, viz.: (*i*) imposing a positive pressure on the head space of a vessel containing eggs immersed in water (Alls *et al.* 1963); (*ii*) drawing and releasing suddenly a vacuum in the head space of a vessel containing eggs immersed in water (Alls *et al.* 1963; Voeten 1965), and (*iii*) directing a water spray on to the wet surface of eggs (Alls *et al.* 1963). The application of these techniques to the dosing of incubating eggs with antibiotics will be considered in *Section D. Practical implications*.

C. A resistance network

Although information permitting a detailed definition of the overall functions of the pores is not available, sufficient progress has been made to allow a discussion of a general concept applicable to the eggs of the domestic hen and probably those of the goose, guinea fowl and duck also. Their shells fall in the category, capped pores, in the classification given in Fig. 1 and the covering material is organic. The fine structure of this type of pore is shown in Plate 1. If such shells are considered as resistance networks, as was done during a discussion of O_2 diffusion across the shell (Tullett & Board 1976), then three major resistances (Fig. 2) can be identified, these being associated with the cuticle (R1), pore canal (R2) and shell membranes (R3) respectively.

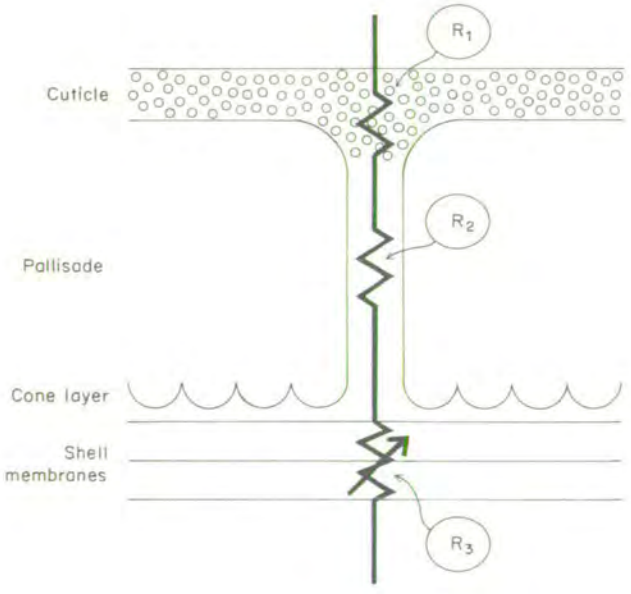

Fig. 2. The major resistances in the avian eggshell.

The available evidence suggests (Rahn *et al.* 1976) that the true (calcitic) shell contains at oviposition a complement of pores (R2) that endows the egg with a certain potential for the flux of respiratory gases and water vapour. The actual rates of flux will be determined by diffusion gradients. Thus the pores may be considered to have a passive role and the actual manifestation of their resistance will be determined by diffusion gradients obtaining in the nest. Although the pores' resistance to gas flow does not change with time, that offered by the shell membranes (R3) does. With the eggs of the domestic hen, for example, their resistance to the diffusion of O_2 is markedly reduced on the 5–7th day of incubation (Lomholt 1976; Tullett & Board 1976). The nature of the changes in the physico-chemical properties of the membranes have not been elucidated. The cuticle on the hens' egg (R1) does not influence appreciably the flux of water vapour (Fig. 3) or gases (Board & Halls 1973*a*; Tullett & Board 1976).

The cuticle's main contribution appears to be as an impediment to the flooding of the pore canals. Thus Board & Halls (1973*a*,*b*) noted that extensive flooding of the pores in the shells of hens', guinea fowl and ducks' eggs occurred with those that were cuticle-less at oviposition or had been rendered cuticle-less by chemical or physical means. Indeed the available evidence supports the contention that the need for protection of flooding of the pores has had a strong selective pressure on shell structure. Thus a study of the fine structure of shells of more than 60 species of birds

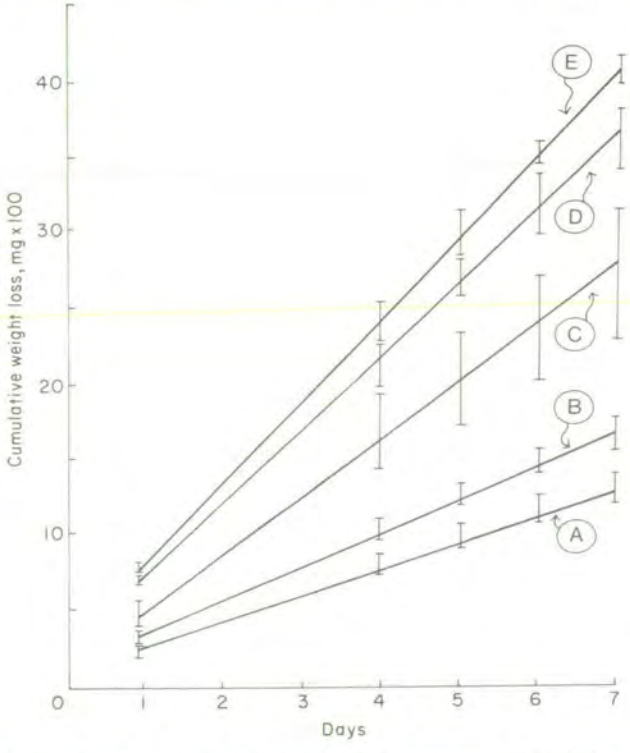

Fig. 3. Water loss by hens' eggs. A, shell made cuticle-less by treatment with ethylene diaminate-traacetic acid; B, control eggs; C, shell at pole pierced with a needle; D, a section (5 × 5 mm) of shell removed without damage to the underlying membrane, and E, shell cracked. The eggs were stored in a desiccator (26°C) containing dry silica gel. Unpublished data of Elizabeth Egglestone and R. G. Board.

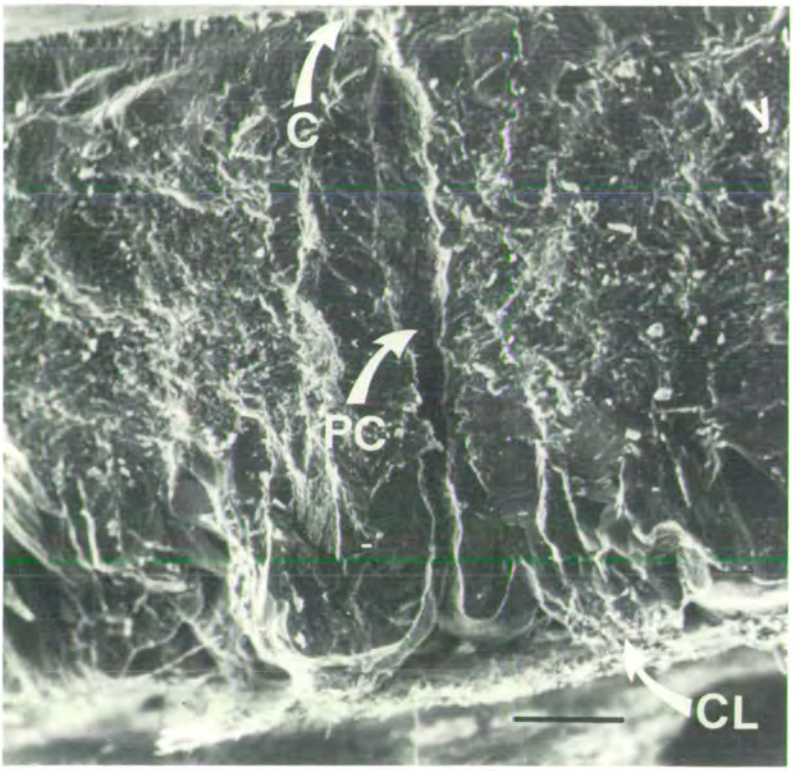

PLATE 1. The fine structure of the pore of the egg of a domestic goose as seen with scanning electron microscopy of a radial section of the shell. PC, pore canal; CL, cone layer; C, cuticle (scale bar, 100 μm = 14 mm).

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showed that well-developed cuticles or covers (Fig. 1) were notable features of eggs of species that nested in wet/dirty places (Board *et al.* 1977). Subsequent studies of eggs laid in very wet nests, *e.g.* those of the Lily Trotter and Great Crested Grebe, have shown remarkable modification of the outer pore orifice; the first mentioned species lays eggs in which the outer orifice of the pore contains a plug and those of the second are covered with a stratum of spheres formed from amorphous material rich in Ca^{2+}, P^{2+} and O_2 (Board & Perrott 1979*b*; Board *et al.* 1980).

Although the available evidence suggests that the pore canal offers a resistance (R2) secondary to that of the cuticle with respect to initial flooding (Board & Halls 1973*a*), its contribution cannot be dismissed entirely. As the inner orifice of the pore canal is separated from the osmotically active albumen by semi-permeable (shell) membranes it might be deduced that, once a pore had been flooded, a thread of water would be pulled along the canal for as long as an egg remained in contact with water and a space (the air cell) to accommodate water uptake obtained. Continuous uptake—to the point of rupture of the shell membranes—can be achieved by removing a small piece of shell. With whole eggs, however, the available evidence is discordant. Board & Halls (1973*a*) were of the opinion that the pore canal imposed sufficient drag to stop the flow of water. Alls *et al.* (1963) demonstrated a progressive increase of antibiotic concentration during the time eggs were submerged in a solution of an antimicrobial agent. Although this can be construed as evidence of liquid movement, the available information does not dismiss the possibility that the increasing concentration of the antibiotic in the egg was the product of a diffusion gradient operating along a static column of water.

The resistances shown in Fig. 2 only operate if the integrity of the shell is maintained. The effect of damage to the shell on water loss is illustrated in Fig. 3. It has been shown, moreover, that when eggs having cracked shells are exposed to a bacterial suspension, the contamination of their contents is much greater than that of eggs with sound shells (Brown *et al.* 1966).

D. Practical implications

Archives recording the culinary practices of our forefathers show that water loss from hens' eggs intended for winter storage was controlled by smearing the shell with butter. The same method is still used but mineral oils are used instead of edible fats. Although water conservation is easily demonstrated, the underlying mechanism has not been elucidated. The concept discussed in this article offers a tentative interpretation; impregnation of the cuticle with oil causes it to become a resistance to the diffusion of water vapour.

The recognition of factors contributing to the flooding of the eggshell with contaminated water led to proposals (*e.g.* Büchli 1967) for the operation of egg-washing machines. The cardinal recommendation was that the temperature of the egg must always be less than that of the washing and rinsing water. In other words, the washing machine must not cause work to be done which would result in pores being flooded.

The control of the transfer of pathogens via the embryo by dosing eggs before incubation with an antimicrobial agent has been attempted for many years. Although some success has attended this practice, the persistence of the research effort in this area is evidence of the failure to devise a fully effective method. Effectiveness can be measured in a number of ways of which variation in the amount—often indexed simply

by determining changes in weight of an egg—of an agent introduced into the individual eggs of a batch is the most common. Too large a variation would imply that some eggs had absorbed a non-effective dose. If such an egg contained a pathogen, the shell fragments, fluff or chick at incubation could be the source of infection of chicken hatched from eggs that had received an adequate dose.

The methods used to introduce antimicrobial agents into eggs are listed in Table 2. All of these were noted in *Section B. Pore flooding*, as examples of means by which work can be done to overcome the water resistance of the shell. Thus the role of the cuticle in impeding the translocation of an antibiotic across the shell is of cardinal importance. Indeed this has been recognized by several workers and they have shown that more effective dosing of an egg can be achieved if the cuticle is removed before the application of an antibiotic (Alls *et al.* 1963; Ekperigin & McCapes 1977). It has been demonstrated by using coloured solutions of antibiotics that some eggs contain relatively few pores that allow the passage of liquid (Alls *et al.* 1964)—such pores are sometimes referred to as patent pores. This was noted also when eggs were dipped in a solution of edicol pea green (Board & Halls 1973*a*). How these few pores differ from the majority is not known. At a more general level, insufficient is known about differences in the 'quality' of cuticle on the individual eggs laid by a bird, to say nothing of variation between breeds. Difference in staining reaction has been used as a crude index of difference (Board & Halls 1973*a*; Ball *et al.* 1975) but more refined methods need to be sought if a better understanding of the major resistance to flooding of the shell is to be achieved.

TABLE 2

Methods used to dose eggs with antimicrobial agents

Method	References*
1. *Temperature differential*† Warm eggs are dipped into a cold solution of an antimicrobial agent. The uptake of the agent is influenced by (a) the temperature differential—the greater the differential, the greater the uptake; (b) duration of dipping, uptake increases with time; (c) number of times the egg is dipped; (d) the cuticle, removal by acid increases uptake, and (e) integrity of the shell, uptake increased if shell is pierced.	Chalquest & Fabricant (1959), Levine & Fabricant (1962), Olson *et al.* (1962), Alls *et al.* (1963, 1964), Ekperigin & McCapes (1977), Mayeda *et al.* (1978)
2. *Vacuum treatment* Eggs in a solution of antimicrobial agent are subjected to a pressure less than atmospheric and then atmospheric pressure imposed suddenly.	Alls *et al.* (1963), Voeten (1965)
3. *Pressure treatment* Eggs in a solution of antimicrobial agent are subjected to a pressure greater than atmospheric.	Alls *et al.* (1963)
4. *Spray* Eggs sprayed with an antimicrobial agent.	Alls *et al.* (1963)

* Selected references only.

† This technique has been widely used to initiate rotting of hens' eggs for laboratory studies and it is notable that factors that accentuate absorption of an antimicrobial agent by an egg, influence also the translocation of bacteria across the shell, viz., the temperature differential (Lorenz *et al.* 1952; Brant & Starr 1962); the period of immersion (Brant & Starr 1962; Hartung & Stadelman 1962), and the treatment afforded the shell—there are numerous reports of an increased incidence of rotting when shells were rubbed with cheesecloth, sandpaper or steel wool before treatment

Recent studies have shown that effective dosing of eggs can be achieved if a hole is scored or drilled in the shell before treatment (Ekperigin & McCapes 1977). It was noted on p. 309 that the shell's efficiency is dependant upon its integrity; thus this new approach could be seen as acceptance of our failure to devise methods that can be relied upon to overcome the resistance offered by an undamaged eggshell. Maybe such an acceptance is premature and it should not be allowed to stifle further research with undamaged eggs, particularly with respect to the form and function of the cuticle. If the thesis of this article—work needs to be done to overcome the water resistance of the shell—is correct then some measure of the amount of work needed to dose all eggs effectively is required. We have attempted to measure the extent of the negative pressure generated by immersing a warm egg in cold water; although a negative pressure could be measured and different rates of decay of this pressure demonstrated, little headway was possible because of the need to use tubing to attach eggs to a pressure transducer (Egglestone & Board, unpublished observations). Some method of remote sensing could be expected to overcome this problem.

If the concept of a resistance network is adopted in an analysis of factors that may contribute to ineffective absorption of chemicals by an egg, then several factors appear to be worthy of study. It was noted in some preliminary studies (Egglestone & Board, unpublished observations) that the amount of water absorbed by eggs could be increased by degassing (by vacuum) the cold water in which eggs were submerged. Could it be that with 'normal' water, the slight negative pressure in the egg is satisfied in part by gas coming out of solution at the cuticle-water interface? The viscosity of the liquid applied to the egg is important also. Thus Board & Halls (1973*a*) demonstrated that liquids of less than 30 dynes penetrated a large number of pores in the hens' eggshell without the need to use any of the methods listed in Table 2. It was noted previously that the wall of the pore canal may impose a resistance (drag) to the inward flow of water even when the pore has been flooded. Thus it would seem that effective uptake of an antimicrobial agent by an egg might be achieved if a hydraulic load was applied to the egg immediately following a process that caused flooding of the pores with a liquid having a low surface tension.

E. Terminology

Studies on water loss from stored eggs, the washing of dirty eggs and the dosing of eggs with antimicrobial agents have generated a limited but probably nebulous terminology (Board & Halls 1973*a*) to which 'patent pores' is the latest recruit. Perhaps the most commonly used term, 'porosity', has been applied not only to the number of pores in a shell—as demonstrated by dye penetration (Fromm 1959), acid etching (Tyler 1953), etc.—but also to some property of the shell, viz., the rate at which water is lost (Tyler 1945), or the electrical conductivity of shells soaked in an electrolyte (Rauch 1952). An attempt to revise the terminology (Board & Halls 1973*a*) can now be considered to have been simply an exercise in semantics because the definitions offered had nothing to do with the biological properties of avian eggshells. If there is a need for technical terms to aid discussion among those who work with the eggs of commerce, then they should be chosen so as not to convey the impression that they describe some biological function of the shell. Such terms are now beginning to emerge from studies both of shell function (*e.g.* Rahn *et al.* 1976) and shell structure (Tullett 1975; Tullett & Board 1977).

3. References

ALLS, A. A., BENTON, W. J., KRAUSS, W. C. & COVER, M. S. 1963 The mechanics of treating hatching eggs for disease prevention. *Avian Diseases* **7**, 89–97.

ALLS, A. A., COVER, M. S., BENTON, W. J. & KRAUS, W. C. 1964 Treatment of hatching eggs for disease prevention—factors affecting permeability and a visual detection of drug absorption. *Avian Diseases* **8**, 245–256.

BALL, R. F., LOGAN, V. & HILL, J. F. 1975 Factors affecting the cuticle of the egg as measured by intensity of staining. *Poultry Science* **54**, 1479–1484.

BOARD, R. G. 1974 Microstructure, water resistance and water repellency of the pigeon eggshell. *British Poultry Science* **15**, 415–419.

BOARD, R. G. 1975 The microstructure of the cuticle-less shell of the domestic hen. *British Poultry Science* **16**, 89–91.

BOARD, R. G. & HALLS, N. A. 1973*a* The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science* **14**, 69–97.

BOARD, R. G. & HALLS, N. A. 1973*b* Water uptake by eggs of mallard and guinea fowl. *British Poultry Science* **14**, 311–314.

BOARD, R. G. & FULLER, R. 1974 Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biological Reviews* **49**, 15–49.

BOARD, R. G., TULLETT, S. G. & PERROTT, H. R. 1977 An arbitrary classification of the pore systems in avian eggshells. *Journal of Zoology, London* **182**, 251–265.

BOARD, R. G., LOSEBY, S. & MILES, V. R. 1979 A note on microbial growth on hen eggshells. *British Poultry Science* **20**, 413–420.

BOARD, R. G. & PERROTT H. R. 1979*a* Vaterite, a constituent of the eggshells of the non-parasitic cuckoos, *Guira guira* and *Crotophagi ani*. *Calcified Tissue International* (in press).

BOARD, R. G. & PERROTT, H. R. 1979*b* The plugged pores of the tinamou (Tinamidae) and jaçana (Jacanidae) eggshells. *Ibis* **121**, 469–474.

BOARD, R. G., PERROTT, H. R., LOVE, G. & SCOTT, V. D. 1980 The fine structure of the eggshells of the Great Crested Grebe (*Podiceps cristatus*). Submitted to *Ibis*.

BRANT, A. W. & STARR, P. B. 1962 Some physical factors relating to egg spoilage. *Poultry Science* **41**, 1468–1473.

BROWN, W. E., BAKER, R. C. & NAYLOR, H. B. 1966 The microbiology of cracked eggs. *Poultry Science* **45**, 284–287.

BÜCHLI, K. 1967 *De invloed van het wassen op de microbiologische houdbaar-heid van het ei*. Instituut voor der Pluimveeteelt 'Het Spelderholt'—Beekbergen Mededeling No. 145.

CHALQUEST, R. R. & FABRICANT, J. 1959 Survival of PPLO injected into eggs previously dipped in antibiotic solutions. *Avian Diseases* **3**, 257–271.

EKPERIGIN, H. E. & MCCAPES, R. H. 1977 Preincubation dipping of turkey hatching eggs, 1. Effect of shell treatment on amount and variability of fluid intake. *Avian Diseases* **21**, 596–604.

FROMM, D. 1959 A rapid method for the determination of eggshell permeability. *Poultry Science* **38**, 171–173.

HAINES, R. B. & MORAN, T. 1941 Porosity of, and bacterial invasion through, the shell of the hen's egg. *Journal of Hygiene, Cambridge* **37**, 453–461.

HARTUNG, T. E. & STADELMAN, W. J. 1962 *Pseudonomas fluorescens* penetration of eggshell membranes as influenced by shell porosity, age of egg and degree of bacterial challenge. *Poultry Science* **42**, 147–150.

LEVINE, P. P. & FABRICANT, J. 1962 Effect of dipping eggs in antibiotic solutions on PPLO transmission in chickens. *Avian diseases* **6**, 72–85.

LOMHOLT, J. P. 1976 The development of the oxygen permeability of the avian eggshell and its membranes during incubation. *Journal of Experimental Zoology* **198**, 177–184.

LORENZ, F. W., STARR, P. B., STARR, M. P. & OGASAWARA, F. X. 1952 The development of *Pseudomonas* spoilage in shell eggs, I. Penetration through the shell. *Food Research* **28**, 24–42.

MAYEDA, B., MCCAPES, R. H. & SCOTT, W. F. 1978 Protection of day-old poults against *Arizona*

hinshawii challenge by pre-incubation streptomycin egg treatment. *Avian Diseases* **22,** 61–70.

NEEDHAM, J. 1942 *Biochemistry and Morphogenesis*. Cambridge: The University Press.

OLSON, N. O., HASH, T. R., HEISHMAN, J. O. & CAMPBELL, A. 1962 Dipping of hatching eggs in erythromycin for the control of mycoplasma. *Avian Diseases* **6,** 191–194.

RAHN, H., PAGANELLI, C. V., NISBET, I. C. T. & WHITTOW, G. C. 1976 Regulation of incubation water loss in eggs of seven species of terns. *Physiological Zoology* **49,** 245–259.

RAUCH, W. 1952 The influence of eggshell porosity on the number of chickens hatched from incubating eggs. *Poultry Science* **31,** 589–594.

TULLETT, S. G. 1975 Regulation of avian eggshell porosity. *Journal of Zoology, London* **177,** 339–348.

TULLETT, S. G., LUTZ, P. L. & BOARD, R. G. 1975 The fine structure of the pores in the shell of the hen's egg. *British Poultry Science* **16,** 93–95.

TULLETT, S. G. & BOARD, R. G. 1976 Oxygen flux across the integument of the avian egg during incubation. *British Poultry Science* **17,** 441–450.

TULLETT, S. G., BOARD, R. G., LOVE, G., PERROTT, H. R. & SCOTT, V. D. 1976 Vaterite deposition during eggshell formation in the cormorant, gannet and shag, and in the 'shell-less' eggs of the domestic fowl. *Acta Zoologica, Stockholm* **57,** 79–87.

TULLETT, S. G. & BOARD, R. G. 1977 Determinants of avian eggshell porosity. *Journal of Zoology, London* **183,** 203–211.

TYLER, C. 1945 The porosity of eggshells, and the influence of different levels of dietary calcium upon porosity. *Journal of Agricultural Science* **35,** 168–176.

TYLER, C. 1953 Studies on eggshells, II. A method of marking and counting pores. *Journal of Food Science and Agriculture* **4,** 266–273.

VOETEN, A. C. 1965 *Control of chronic respiratory disease in chickens by egg dipping in a solution of tylosin tartrate*. Ph.D. Thesis, University of Utrecht.

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The Properties and Classification of the Predominant Bacteria in Rotten Eggs

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SUMMARY. A collection of 226 strains of bacteria was assembled from eggs which had rotted on the premises of the producer and from others which had been allowed to rot in the laboratory at 10, 20 or 30°. A majority of the eggs had a mixed infection. All but 8 of the isolates were Gram negative rods. The predominant types were *Alcaligenes faecalis*, *Aeromonas liquefaciens*, *Proteus vulgaris*, *Cloaca* spp., *Citrobacter* sp. and *Pseudomonas fluorescens*. A nonpigmented pseudomonad could not be identified with any of the species included in *Pseudomonas*.

A MIXED INFECTION dominated by Gram negative bacteria appears to be a common feature of rotten eggs (Pennington, Jenkins, St. John & Hicks, 1914; Miles & Halnan, 1937; Haines, 1938; Alford, Holmes, Scott & Vickery, 1950; Miller & Crawford, 1953; Florian & Trussell, 1957). Following the investigations of Miles & Halnan (1937) it became customary to inoculate newly laid eggs with pure cultures in order to determine which of the isolates was responsible for the changes observed in the original rot. The organisms responsible for these changes have been characterized in detail but those incapable of changing the appearance of the yolk or white, the 'secondary invaders' of Florian & Trussell (1957), have been largely ignored. This bias was perhaps permissible when eggs were stored for long periods because of seasonal fluctuations in production. The development of intensive methods of poultry husbandry has now tended to spread production more evenly over the year and the recent expansion in the manufacture of egg products in the United Kingdom has permitted the rapid disposal of surplus eggs. These products can be heavily contaminated (Brooks & Taylor, 1955), mainly with Gram negative bacteria (Wrinkle, Weisser & Winter, 1950), but the relative importance of the sources of contamination has not been determined. A possible and, until now, rarely suspected source has been suggested by some recent observations (Board, 1964; Board, Ayres, Kraft & Forsythe, 1964). These workers found that eggs infected with nonproteolytic, nonpigmented organisms showed little indication of infection. It is doubtful, therefore, whether eggs contaminated only with the 'secondary invaders' of Florian & Trussell (1957) or some of the organisms recovered by Wrinkle *et al.* (1950) would be detected by the 'look and smell' test which is used routinely by manufacturers of egg products. Such eggs have been recovered from egg breaking plants (Pennington *et al.*, 1914; Johns & Bérard, 1945, 1946) and heavily contaminated eggs which on cursory examination differed from sterile eggs only in the possession of an off flavour or off odour have been a frequent cause of complaint from the housewife (Turner, 1927; Spanswick, 1932; Levine & Anderson, 1932; Richard & Mohler, 1950). This evidence together with the

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rapidly changing pattern of egg production suggests that all the commonly occurring contaminants of the contents of eggs should be considered important.

Previous studies in the United Kingdom have been concerned with imported or with stored home produced eggs (Miles & Halnan, 1937; Haines, 1938). In the present investigation eggs which had rotted on the premises of the producer or under controlled conditions in the laboratory were examined. Many of the isolates included in the present study have earlier been used to follow the course of infection in the egg (Board, 1964) and to study bacterial growth on the shell membranes *in vitro* (Board, 1965). The present report is concerned with the properties and classification of the organisms numerically predominant in rotten eggs.

Materials and Methods

Nutrient broth and nutrient agar

Nutrient broth contained (% w/v): peptone (Evans), 0·25; Bacto-peptone (Difco), 0·25; Lab-Lemco (Oxoid), 0·5; NaCl, 0·25; in tap water; pH 7. Nutrient agar contained in addition 1·5% of agar (Davis, New Zealand). These media were sterilized by holding momentarily at 128° (22·5 lb/in^2 steam pressure).

Rotten eggs

These were obtained from two egg collecting centres in the South East of Scotland and one in the South of England. The eggs had rotted on the premises of the producers and were rejected during candling immediately on receipt at the centres. Eggs induced to rot under controlled conditions were also examined. First quality eggs (laid by White Leghorns), which had been badly soiled with faeces and/or deep litter material, were washed in tap water and then held for several hours at 37° before being returned to the wash water (18–20°). Bacterial penetration of the shell is known to be assisted by the contraction of a warm egg on cooling (Haines & Moran, 1940). The eggs were allowed to soak for 20 min. They were then placed in screw capped glass jars, the bottom of which were lined with moist cotton wool, and stored at 30, 20 or *c.* 10°. Three separate batches of eggs were treated in this manner: they were candled frequently and those showing deterioration were examined bacteriologically. A total of 133 eggs was examined.

Isolation of micro-organisms from rotten eggs

The eggs were wiped with alcohol and placed on a pipe clay triangle the arms of which had been bent through 90° so that the triangle now stood on three legs. The shell was flamed before being pierced with a sterile scalpel and the contents were poured into a Petri dish. The features of the contents were noted, including the appearance in ultraviolet light. A loopful of the material was streaked on nutrient agar containing 0·3% (w/v) of yeast extract (Yeastrel) and glucose, sterilized by filtration, at a final concentration of 0·1% (w/v). Two streak plates were prepared from each rot, one of which was incubated aerobically at 27° and the other was covered with 5 ml of (g/l): cysteine hydrochloride, 0·5; thioglycollic acid, 0·05; agar (Davis, New Zealand), 15; resazurin, 0·5 mg; pH 7·2. This plate was incubated in a McIntosh and Fildes jar containing an atmosphere of 5 parts of CO_2 and 95 parts of H_2. After 7 days

representatives of each type of colony were transferred to nutrient broth. Their purity was ensured by replating on at least three occasions. Selected colonies developing during anaerobic incubation were stab-inoculated into nutrient agar containing 0·5% (w/v) of glucose and the donor colony was tested for catalase activity.

Maintenance of stock cultures

One set of cultures was maintained under mineral oil on nutrient agar slopes at room temperature, and another was stored in nutrient broth at 4°. This latter set was subcultured at 6 monthly intervals, at which time the purity of the culture was checked and fresh broth cultures were prepared for laboratory use.

Characterization of isolates

An 18 h culture in nutrient broth was used as the inoculum in all experiments and all tests were made at 27°, except in the case of nutrient gelatin which was incubated at 22°. Recordings were made at frequent intervals during the first 14 days and finally on the 28th day.

Flagella staining (Rhodes, 1958). Preparations were made from nutrient agar slope cultures after 12, 24, 48 and 96 h.

Fermentation tests. The medium of Hugh & Leifson (1953) was used routinely, but bacteria which produced either no change in pH or an alkaline reaction in this medium were retested in the medium of Board & Holding (1960). Both inoculated tubes of basal medium were incubated along with medium containing the fermentation substrate. The latter was sterilized by filtration, except with the primary alcohols which were held for 15 min at 100°, and then added to the liquefied medium cooled to 45°. The substrates were normally used at a final concentration of 0·5% (w/v) or, with the primary alcohols, 0·5% (v/v). Exceptions to this are noted in the text. Stab inoculations were made into 10 ml of medium in 15×180 mm tubes.

Acetoin production and M.R. test. Cultures in 1% (w/v) peptone (Evans) water containing glucose (0·5%, w/v) were examined by Barritt's (1936) method and the pH of the medium was tested with methyl red.

Starch hydrolysis. Plates of nutrient agar containing 1·0% (w/v) of soluble starch were inoculated on the surface and after incubation were flooded with a solution of Lugol's iodine.

H_2S production. Filter paper soaked in lead acetate was suspended over 1% (w/v) peptone (Evans) water containing a piece of heat coagulated egg white.

Deamination of phenylalanine. Ferric chloride was used to test for the presence of phenylpyruvic acid in the water of syneresis at the base of nutrient agar slopes containing (±) phenylalanine (0·4%, w/v). In the case of doubtful reactions a cell suspension prepared from a nutrient agar slope culture (Henriksen & Closs, 1938) was tested by the method of Stewart (1959).

Indole. Cultures in tryptone (Difco) broth (1·0%, w/v) were tested by the method of Roessler & McClung (1943).

Arginine test. Medium 2A of Thornley (1960) was dispensed in 3 ml amounts in 5 ml screw capped bottles. After inoculation the remaining free air space was filled with sterile soft paraffin.

Urea hydrolysis. Those bacteria which produced an alkaline reaction in the medium of Christensen (1946) were recorded as urease positive.

Nitrate reduction. Cultures in peptone (Evans) water (1%, w/v) and KNO_3 (0·1%, w/v) were tested for nitrite and, if negative, nitrate.

Casein hydrolysis. Plates of nutrient agar containing 10% (v/v) of skim milk were inoculated on the surface and after incubation they were flooded with acid mercuric chloride.

Oxidase test. A loopful of cells from a nutrient agar slope culture was placed on filter paper moistened with 1% (w/v) aqueous solution of *p*-amino-N:N-dimethyl aniline (laboratory reagent grade, British Drug Houses Ltd). The development of a blue colour within 1 min was recorded as a positive reaction.

Production of lecithinase. Egg yolk was prepared by the method given in the *Manual of Methods* (1957). Lecithinase activity was tested by inoculating either nutrient agar or nutrient broth containing 10% (v/v) of egg yolk.

Chelated gluconate medium (Paton, 1959). This was used to demonstrate the production of fluorescent pigments by the pseudomonads.

Growth temperatures. Inoculated nutrient agar slope cultures were incubated at 4° in a beaker of water in a cold room or at 10, 20, 30 or 40° in thermostatically controlled water baths.

Results

Of the 133 eggs reported on in the present communication, 70 were obtained from commercial channels and the remainder had been treated in the laboratory.

TABLE 1

The preliminary grouping of 226 strains of bacteria isolated from rotten eggs

Type of organism	No. of isolates	Utilization* of		Hydrolysis† of urea	Fluorescence of litmus milk cultures in ultraviolet light
		Glucose	Lactose		
Alcaligenes spp.	52	−	−	−	−
Nonpigmented pseudomonads	24	*O*	*O*	−	−
Fluorescent pseudomonads	39	O	−	−	+
Proteus spp.	15	F	−	+	−
Coli-aerogenes‡ bacteria	75	F	F or −	−	−
Ungrouped Gram negative rods	13	d	d	d	d
Streptococci	2	NT	NT	NT	NT
Micrococci	4	NT	NT	NT	NT
Gram positive rods	2	NT	NT	NT	NT

* Tested with the medium of Hugh & Leifson (1953) or Board & Holding (1960).

† Tested with the medium of Christensen (1946).

‡ Strains of *Aeromonas* included in this group.

+, all strains positive; −, all strains negative; d, different reactions within a group; *O*, small amount of acid produced in presence of oxygen only; O, large amount of acid produced in presence of oxygen only; F, acid produced in presence or absence of oxygen; NT, not tested.

Subsequent experiences with rotten eggs derived from divers sources in the United Kingdom, U.S.A. and Lebanon have indicated that this was sufficiently extensive to include a normal and representative range of both bacterial types and egg rots. Neither bacteria which grew only in the absence of gaseous oxygen, nor any moulds or yeasts were found, the absence of the latter two groups being confirmed by microscopic examination of infected material stained with polychrome methylene blue.

The preliminary separation of the 226 isolates was based on the following tests: shape, reaction to Gram's stain, reaction in litmus milk, action on glucose, lactose and urea, oxygen requirements and examination of cultures on nutrient agar or in litmus milk under ultraviolet light. Gram negative bacteria were dominant (Table 1), and there were no appreciable differences in the types of organisms isolated from eggs of commercial origin and those which had rotted in the laboratory (Table 2). In the latter instance, however, the temperature of storage appeared to exert an influence. Eight strains of *Proteus vulgaris* were recovered from eggs held at 30°, 1 from eggs held at 20° but none from those held at 10°. Fluorescent pseudomonads were not recovered from eggs held at 30°. Further evidence of the selective influence of temperature has been given elsewhere (Board *et al.*, 1964; Board & Ayres, 1965).

TABLE 2

The incidence of different types of Gram negative bacteria in rotten eggs from various sources

Type of organism	No. of times organisms isolated from eggs rotted				
	in the laboratory at			commercially from	
	10°	20°	30°	Scottish sources	English sources
Alcaligenes spp.	5	26	6	12	3
Nonpigmented pseudomonad	10	8	3	15	3
Fluorescent pseudomonad	4	5	0	9	6
Proteus spp.	0	1	8	6	0
Coli-aerogenes* bacteria	7	9	7	40	12
Total no. of eggs examined	27	19	17	56	14

* Strains of *Aeromonas* included in this group.

Representatives of the various groups listed in Table 1 were selected for detailed examination. In making this selection, care was exercised to ensure that the collection contained organisms which had been isolated from all the sources listed in Table 2. In all instances the properties of the organisms were tested on at least 2 occasions during the 2 years following isolation. Most of the results are summarized in Table 3. Additional details and the identification of these groups are discussed below.

Alcaligenes *species*

A homogenous group was formed by the 9 representative strains isolated. They were inert in the more commonly used biochemical tests, but some of the following characteristics proved useful adjuncts in recognizing them. Skim milk containing either litmus or bromthymol blue became alkaline within 72 h and remained so for 28 days; respirometer studies and tests with Fehling's reagent on growing cultures

TABLE 3

Properties of Gram negative bacteria isolated from rotten eggs

	Alcaligenes spp.	Nonpigmented pseudomonads	Fluorescent pseudomonads	*Proteus* spp.	Coli-aerogenes bacteria		Aeromonads
					A	B	
No. of isolates examined	9	10	9	6	14	7	6
Position of flagella	Peritrichous	Polar	Polar	Peritrichous	Peritrichous	Peritrichous	Polar
Action on glucose	−	A	A	F	FR	F	FR
Action on lactose	−	A	−	−	9FR, 5−	F	4FR, 2−
Hydrolysis of starch	−	−	−	−	−	−	+
Acetoin formation	−	−	−	−	+	−	5+, 1−
Methyl red test	−	−	−	+	−	+	−
Gelatin liquefaction		+	6+, 3−	+	6+, 8−	−	+
H_2S production	−	++	−	++	+	+	++
Indole*	−	−	−	+	−	1+, 6−	+
Arginine test*	−	−	+	−	−	−	+
Urea hydrolysis*	−	−	−	+	−	−	−
Deamination of phenylalanine	−	−	−	+	−	−	−
Oxidase test	+	−	+	−	−	−	+
Action on KNO_3	−	−	d	NO_2 produced	NO_2 produced	NO_2 produced	NO_2 produced
Egg yolk reaction	−	−	5+, 4−	−	6+, 8−	−	+
Range of growth temperatures (°C)	20–30	4–30	4–30	20–40	4–30	4–30	4–30

A, acid produced, aerobically only; F, acid produced in both presence and absence of gaseous oxygen; R, reversion of pH to neutral reaction; −, negative reaction; +, positive reaction; ++, strong positive reaction; d, different reactions among the organisms tested (see Table 4); numerals, number of strains responding as indicated; *, for method of testing see text.

failed to demonstrate glucose utilization, results which confirmed those obtained from conventional fermentation tests; acidic substances were formed in an oxidative breakdown of ethanol, propanol or butanol (tested in the medium of Board & Holding, 1960) but no clearing was seen in the ethanol-$CaCO_3$ medium of Shimwell, Carr & Rhodes (1960); in media in which ammonia was the sole source of combined nitrogen, growth occurred when the carbon source was ethanol but not when it was glucose or citrate; no growth occurred when ethylamine or methylamine was the sole source of carbon and nitrogen; there was no detectable action on egg yolk, potato or glycerol (tested for acid production and, using Benedict's reagent, the formation of hydroxyacetone) and no growth occurred in nutrient broth at pH 4·5 (adjusted with HCl).

Although the 9 strains selected for detailed examination were found to be homogenous from the biochemical standpoint, a characteristic variation in the form of the colony was noted. This property may have been responsible for the isolation of such large numbers of these organisms. Some cultures produced equal numbers of two types of colony, namely, (i) a shiny round raised colony with a regular border, and (ii) a round raised shiny plateau encircled by a flat area with an irregular edge. Both of these were seen with *Alcaligenes faecalis* NCIB 8156; with other cultures one or other of these types of colony was numerically predominant.

The bacteria isolated in this study have been identified with *Alc. faecalis*, a conclusion supported by comparison with Conn's culture of *Alc. faecalis* (NCIB 8156).

Fluorescent pseudomonads

Table 4 shows the biochemical differences that existed among the 9 isolates chosen for detailed examination; they were all identified with *Ps. fluorescens* as defined by Rhodes (1959).

TABLE 4

Some properties of fluorescent pseudomonads isolated from rotten eggs

Strain no.	Response to			
	Gelatin liquefaction test	Action on KNO_3	Lecithinase test	Action on litmus milk
G3/1	+	Reduction, gas formed	+	Digested
G12/1	+	−	+	Digested
CF3	+	Reduction, gas formed	+	Digested
CF21*	+	Reduction, gas formed	+	Digested
CF23/1	+	Reduction, gas formed	+	Digested
ED9/2	+	Reduction, gas formed	+	Digested
G10	−	−	−	Alkaline
G29/1	−	−	−	Alkaline
Cw6/3	−	−	−	Alkaline

* On original isolation this strain produced a brown pigment which was slowly lost during maintenance on laboratory media.

Proteus *species*

The 6 representatives of this group had the following properties in addition to those given in Table 3. All the strains fermented sucrose and 5 of the 6 fermented mannitol, dulcitol and inositol; none of the strains produced transferable growth in a mineral medium containing either glucose or citrate; they had no detectable action on egg yolk; a brown pigment was formed on nutrient agar containing additional tyrosine after prolonged incubation at room temperature; they produced proteus type (swarming) growth on nutrient agar. The 6 strains are considered to be *Proteus vulgaris* Hauser.

Coli-aerogenes *group*

The results of the test for acetoin formation and the methyl red reaction were used to separate the isolates into A and B of Table 3. Group A has been identified with *Cloaca* (*Report*, 1958) and two main types were recognized: (i) a non-lactose fermenting, proteolytic group which produced a heavy scum on nutrient broth containing egg yolk, and a soft clot with slight acidity and marked digestion in litmus milk, and (ii) a lactose fermenting group which had no detectable action on egg yolk and produced only an acid reaction in litmus milk.

Strains of group B (Table 3) have been identified with *Citrobacter* (*Report*, 1958).

Aeromonads

Strains of this group were not recognized in the preliminary work and it was not until flagella staining had been done that the present collection was obtained. The identification of these with *Aeromonas* was supported by studies in which they were compared with *Ps. fermentans* von Wolzogen Kühr strain L417, which was obtained from the late Dr. B. P. Eddy, and *Aer. hydrophila* (NCTC 7810). The strains isolated in the present study have been identified with *Aer. liquefaciens* as defined by Eddy (1960). Three of our isolates were examined by the late Dr. B. P. Eddy and he was in agreement with the above identification (Eddy, 1962).

Nonpigmented pseudomonads

A homogeneous group was formed by the 10 isolates. These organisms will not grow in the absence of gaseous oxygen and have 1–3 flagella attached to one pole of the cell, an arrangement which did not change during incubation for 96 h.

On nutrient agar, a smooth circular raised colony of butter-like consistency was generally formed although occasionally an irregularly outlined rugose colony developed. An unsuccessful attempt was made to find the reason for this variation. Young cultures on nutrient agar were colourless but the growth became slightly yellow after prolonged incubation at room temperature. This pigment could be most easily seen on media containing $CaCO_3$. This pale yellow colour was also evident in the packed cells when an 18 h nutrient broth culture was centrifuged. No pigment was formed on the gluconate medium of Paton (1959) which did not support growth unless supplemented with, e.g., yeast extract.

Good growth was obtained in all the common laboratory media. In media in which ammonia was the sole source of nitrogen, no growth occurred with glucose, gluconate.

citrate or ethanol as the carbon source. None of the following could serve as a source of both carbon and nitrogen: ammonium lactate, methylamine or ethylamine. Growth was obtained when a glucose mineral medium was supplemented with yeast extract but the amount of growth (visual estimation) appeared to be determined by the concentration of yeast extract.

Viability on nutrient agar was quickly lost at room temperature when the culture was covered with mineral oil, but it was maintained satisfactorily in nutrient broth subcultured at 6 monthly intervals and stored at 4° or room temperature.

TABLE 5

The influence of different media on the demonstration of glucose utilization by a nonpigmented pseudomonad

Basal medium	Concn glucose (%, w/v)	Reaction at 27° after 1 day	2 days	3 days	7 days
Hugh & Leifson's (1953)	0	k	K	K	K
	0·05	k	–	–	–
	0·10	k	–	–	a
Hugh & Ryschenkow's (1961)	0	K	K	K	K
	0·05	K	K	K	K
	0·10	K	K	K	K
Board & Holding's (1960)	0	–	k	k	k
	0·05	–	–	–	A
	0·10	–	–	–	A

–, no detectable change in the colour of bromothymol blue; k, weak alkaline reaction; K, strong alkaline reaction; a, weak acid reaction; A, strong acid reaction.

No clear cut reactions were noted when the medium of Hugh & Leifson (1953) was used to test for the utilization of glucose. There was a pronounced alkaline reaction in the basal medium alone and it was inferred that this reaction masked much of the acid which arose from the utilization of glucose. A medium was devised (Board & Holding, 1960) in which 0·05% (w/v) of yeast extract was the source of organic nitrogen. No pronounced alkaline reaction developed in this medium but when it contained glucose an acid reaction was registered by the seventh day of incubation (Table 5). The demonstration of the disappearance of glucose from a growing culture provided additional evidence of the ability of these organisms to utilize glucose. Confirmation was also obtained by standard manometric techniques from which (Fig. 1) it will be seen that glucose was used by cells harvested from nutrient agar as well as by those obtained from nutrient agar containing 0·5% (w/v) of glucose. Acid reactions, detectable only in the medium of Board & Holding (1960), were obtained with fructose, xylose, lactose, ethanol, propanol and butanol. No clearing occurred during growth on the ethanol-$CaCO_3$ medium of Shimwell *et al.* (1960). A strong acid reaction developed in the media of Hugh & Leifson (1953) and Hugh & Ryschenkow (1961) containing either arabinose or maltose.

No change in pH was seen in skim milk containing either litmus or bromothymol blue but the milk was slowly digested from the top downwards and a soft clot formed at the bottom of the tube. A very characteristic almond-like (nutty) odour was

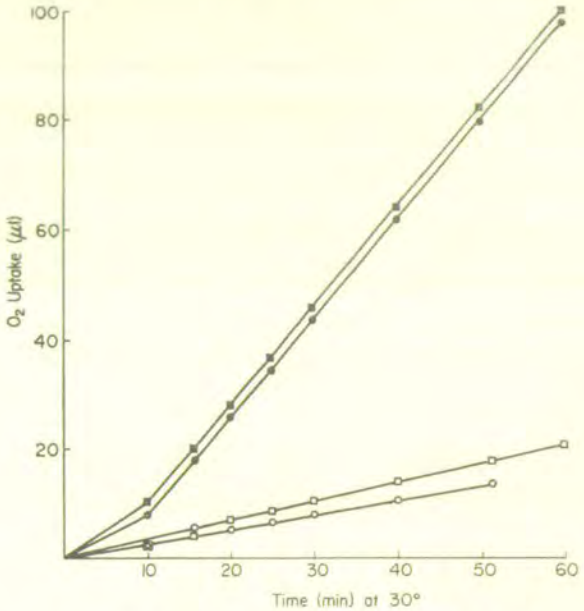

Fig. 1. Oxygen uptake by a nonpigmented pseudomonad in the presence of glucose. Warburg flasks contained washed cells (equivalent to 1·25 mg dry wt/ml) 1·0 ml; M/15 phosphate buffer (pH 7·0) 1·0 ml; substrate (0·1 M glucose or M/15 phosphate buffer for endogenous respiration) 1·0 ml; KOH in central well. Responses of an 18 h nutrient agar culture with glucose (closed squares); control (open squares). Responses of an 18 h glucose nutrient agar culture with glucose (closed circles); control (open circles).

produced in milk. The odour was also detected in infected eggs and on a number of media containing proteins, particularly those derived from the egg. This property did not decline during a 3 year period in which these organisms were maintained on laboratory media.

These organisms digested gelatin, casein and heat coagulated egg white. The rate of digestion was slow as judged by the speed of liquefaction in nutrient gelatin at 22° where only about a third of the gelatin was liquefied on the 28th day. No liquefaction occurred when nutrient gelatin, either with or without glucose, was covered with mineral oil or water agar containing reducing substances. Hydrogen sulphide was formed during the digestion of proteins, particularly in peptone water containing heat coagulated egg white where at 27° it was rapid. The organisms had no detectable action on egg yolk, potato or glycerol (tested for acid production and the formation of hydroxyacetone) and they did not grow in nutrient broth adjusted to pH 4·5 with HCl.

Bacteria of this group were considered to have biochemical properties similar to those which Florian & Trussell (1957) recorded as either polarly or peritrichously flagellate and identified with *Alc. bookeri*. The properties of one of their isolates, a peritrichously flagellate organism obtained from Dr. J. A. Garibaldi, set out in Table 6, show that this organism had few properties in common with the nonpigmented

TABLE 6

Comparison of the biochemical reactions of the nonpigmented pseudomonads with some other bacteria

Reagent	Response of			
	Nonpigmented pseudomonads (10 isolates from eggs)	*Pseudomonas maltophilia* (NCIB 9201, 9202, 9203, 9204)	*Pseudomonas fragi* (NCIB 8542)	*Alcaligenes bookeri* (29d from Dr. J. A. Garibaldi*)
Glucose	a	a	A	–
Fructose	a	a	NT	NT
Maltose	A	A	NT	NT
Lactose	a	a	NT	NT
Arabinose	A	–	NT	NT
Xylose	a	–	NT	NT
Ethanol	a	–	NT	a
Oxidase test	–	–	+	+
Gelatin liquefaction	+	+	+	–
Arginine test	–	–	+	NT
Litmus milk	No pH change Digestion Soft clot Almond-like odour (strong)	No pH change Digestion Soft clot Almond-like odour (weak)	No change No odour	Reduction of litmus No odour
Nutrient broth	Granular turbidity	Uniform turbidity	NT	NT

* Dr. Garibaldi obtained this strain from Florian & Trussell (1957).

–, no detectable action; a, acid reaction detectable only in the medium of Board & Holding (1960); A, acid reaction detectable in the medium of Hugh & Leifson (1953); NT, not tested.

pseudomonads isolated in the present investigation. The available evidence suggests that it belongs to the genus *Alcaligenes* but since it fails to digest gelatin it is distinguishable from *Alc. bookeri*.

The organisms discussed in this section have been identified with *Pseudomonas* as defined in *Bergey's Manual*, 7th ed. (1957). Attempts at specific identification resulted in *Ps. fragi* (Hussong, Long & Hammer, 1937) and *Ps. maltophilia* (Hugh & Ryschenkow, 1960, 1961) being chosen for comparative purposes, and the findings on this basis are summarized in Table 6. Our organisms and *Ps. maltophilia* shared a number of properties, in particular the action on litmus milk and sugars, and in glucose utilization *Ps. maltophilia* gave results similar to those shown in Table 5. Certain differences became evident (Table 6) but in the present state of knowledge their taxonomic significance cannot be assessed. In the meantime the organisms isolated in the present study will be referred to as 'pseudomonad E'.

Changes in eggs produced by different types of bacteria

Representatives of the groups discussed above were tested for their ability to change the appearance of the contents of the hen's egg by inoculating 4 eggs (Board, 1964) with 0·1 ml of a nutrient broth culture and noting the appearance of the yolk and the white after 42 days at 27°. In addition the purity of the culture was checked by subculturing a loopful of the albumen on nutrient agar. The results (Table 7) show that profound changes occurred in eggs inoculated with *Aer. liquefaciens*, *Pr. vulgaris*,

TABLE 7

Changes produced in eggs after 42 days at 27° by bacteria isolated from rotten eggs

Organism	No. of strains tested	Changes occurring in egg	Type of rot*
Aeromonas liquefaciens	8	Grey watery albumen; gelatinous yolk blackened throughout	Black, type 1‡
Proteus vulgaris	4	Dark brown albumen; dark brown mealy yolk	Black, type 2‡
Pseudomonas fluorescens	5	Mealy yolk, fluorescent green albumen	Pink*
	4	Fluorescent green pigment in albumen	Fluorescent green*
Nonpigmented pseudmonad	4	Gelatinous amber-like yolk striped with olive green pigment	Green*
Cloaca spp.	6 †	Yolk encrusted with custard-like material	Custard*
	8 ‖	Occasional faint turbidity in the albumen	
Citrobacter spp.	7		
Alcaligenes faecalis	4		

* The rots were identified according to descriptions given by: *, Florian & Trussell (1957); ‡, by Haines (1939); †, lecithinase produced; ‖, lecithinase not produced.

Ps. fluorescens, the nonpigmented pseudomonad and those strains of *Cloaca* which produced lecithinase. Eggs inoculated with *Alc. faecalis*, *Citrobacter* or the non-lecithinase producing strains of *Cloaca* did not differ from uninoculated eggs which had been incubated for a similar period of time. Board (1964) previously noted that the course of infection, as judged by changes in the size of the microbial populations in the shell membranes and the albumen, is similar for both groups of organisms. Marked changes in the appearance of the contents is produced only by organisms which possess one or more of the following properties: chromogenesis, proteolytic activity, the ability to produce H_2S or attack lecithin.

Discussion

The appearance of the yolk and white of rotten eggs derived from commercial sources appears to have been the sole feature of interest to many of the early investigators (e.g. Benjamin, 1914); the properties of the bacterial contaminants were largely ignored. Such an approach rested on the assumption that the various types of rots represented stages leading to the final state of decomposition—the black rot—and that this development occurred independently of the metabolic attributes of the bacterial contaminants. Although the majority of rotten eggs contain a mixed infection of Gram negative bacteria, it is known (Miles & Halnan, 1937; Haines, 1938, 1939) that specific organisms are responsible for the characteristic features of the commonly occurring rots. These organisms possess one or more of the following properties (Board, 1964): chromogenesis, proteolytic activity, ability to produce H_2S, attack lecithin. The terms 'adventitious contaminants' (Haines, 1939) or 'secondary invaders' (Florian & Trussell, 1957) have been applied to those isolates which do not possess any of these properties. These terms have an erroneous implication because

many of them can form large populations in eggs without producing easily detected signs of infection (Board, 1964). It is noteworthy that such eggs have been recovered from egg breaking plants (Pennington *et al.*, 1914; Johns & Bérard, 1945, 1946) and that the causative organisms are normal constituents of egg products (Wrinkle *et al.*, 1950). Thus it would appear that at this time when the manufacture of egg products is increasing any organism derived from rotten eggs is worthy of a full characterization.

A new species, *Pr. melanovogenes*, was created (Miles & Halnan, 1937) for organisms which caused a characteristic black rot in eggs imported from South Africa. Later these organisms were assigned to the genus *Aeromonas* (Miles & Miles, 1951) and identified (Eddy, 1960, 1962) with *Aer. liquefaciens*. They appear to be infrequent contaminants of commercial eggs. A few strains were recovered from Canadian (Florian & Trussell, 1957) and American eggs (Board *et al.*, 1964) and 6 were isolated in the present study.

Alford *et al.* (1950) claimed that black rots in eggs held at 20° or above are normally caused by strains of *Proteus*, an assertion which was not supported by information concerning the properties of the organisms. A similar view was held by Haines (1938) but the evidence upon which it was based does not allow the reader to separate *Proteus* from *Aeromonas* or other nonlactose fermenting organisms. It is noteworthy that none of the organisms of Haines hydrolyzed urea rapidly. On the basis of urea breakdown under conditions which were not specified, Florian & Trussell (1957) identified several of their isolates with *Pr. vulgaris* but it should be noted that the organisms did not produce the type of rot normally associated with that species. The changes which they produced in eggs appear to be similar to those effected by the lecithinase forming *Cloaca* strains isolated in the present study. When the medium of Christensen (1946) was used to demonstrate the hydrolysis of urea, many of the last mentioned organisms slowly produced an alkaline reaction in the 2–3 days following inoculation. Apparently the present report is the first in which the occurrence of *Pr. vulgaris* in rotten eggs has been supported by satisfactory evidence.

Nonlactose fermenting organisms other than *Proteus* have been isolated from eggs on a number of occasions. Tissier (1926) created a new species, *Bacillus thiaminophilus*, for organisms which in addition to their failure to ferment lactose were indole negative, did not liquefy gelatin, did not agglutinate with antiserum prepared against *Salmonella* or *Shigella* and grew poorly at 37°. Organisms with essentially the same properties were isolated from French eggs by Lagrange (1935). Florian & Trussell (1957) assigned their nonlactose fermenting organisms to *Paracolobactrum*, as defined in *Bergey's Manual*, 6th ed. (1948), and recognized the species *Paracol. intermedium* and *Paracol. aerogenoides*. It is worthy of note that all these organisms have many properties in common with the nonlactose fermenting isolates assigned to *Cloaca* in the present study.

Coli-aerogenes organisms appear to be common contaminants of rotten or tainted eggs. In the investigations of Pennington *et al.* (1914) *Bact. coli commune* was isolated from 55%, *Bact. lactis aerogenes* from 30% and a mixture of the 2 from the remaining 15% of faults referred to as 'sour eggs'. The latter appeared normal when candled but a sour odour was given off by the contents. Florian & Trussell (1957) identified their isolates with *E. freundii*, *E. intermedia* or *Aer. cloacae*. Strains of the latter together

with *Aer. aerogenes* were recovered from tainted eggs by Richard & Mohler (1950). Of the 11 coli-aerogenes characterized in detail by Haines (1938), 9 appear to be essentially the same as the organism identified with *Citrobacter* in the present study; the other 2 could be assigned to *Cloaca*.

Strains of *Serratia marcescens* have been recovered occasionally from rotten eggs (Bennetts, 1931; Alford *et al.*, 1950) but these organisms were not encountered in the present study.

Many workers have recovered pigment producing pseudomonads from eggs. It would appear that the pyocyanine producers are of rare occurrence: the wastage of eggs due to *Ps. pyocyanea* was reported by Platt & Anderson (1939) and the same species was isolated from a black rot by Miles & Halnan (1937). From faults referred to as 'green whites' or 'grass eggs', Pennington *et al.* (1914) isolated organisms which they identified with *Ps. syncyanea* Migula, and Pavarino (1929) isolated *B. fluorescens liquefaciens*. Organisms identified with *Ps. fluorescens* have been recovered from rotten or tainted eggs by several workers (Alford *et al.*, 1950; Richard & Mohler, 1950; Florian & Trussell, 1957). It is of interest to note that a comparative study by Szybalski (1950) of polarly flagellate organisms derived from tainted eggs has revealed that a fluorescent green pigment is formed by some of the strains which had been identified with *Achromobacter perolens* Turner and with *Ps. graveolens* Levine and Anderson. In the course of an investigation of imported and home produced eggs, Haines (1938) gave a detailed characterization to 29 strains of fluorescent pseudomonads but considered that the unsatisfactory nature of the existing differential criteria did not permit specific identification. These organisms had many properties in common with those which we identified with *Ps. fluorescens* as defined by Rhodes (1959).

Alford *et al.* (1950) claimed that nonpigmented pseudomonads are one of the main causative agents of 'colourless rots' but the properties of the organisms were not given. Many strains of nonpigmented pseudomonads were included in the present study and these produced characteristic changes when seeded in fresh eggs. These changes appeared to be essentially the same as those produced by some of the organisms which Florian & Trussell (1957) identified with *Alc. bookeri*. As mentioned previously, there is doubt concerning this identification particularly as polarly and peritrichously flagellate organisms were assigned to the same species.

Strains of *Alcaligenes* and *Achromobacter* have been isolated from rotten eggs (Miles & Halnan, 1937; Alford *et al.*, 1950; Richard & Mohler, 1950; Florian & Trussell, 1957) but the lack of essential detail does not allow the validity of the identifications to be checked. In the present study, several strains of *Alc. faecalis* were studied and their identity was checked by comparison with Conn's strain of *Alc. faecalis* (NCIB 8156).

It is clear from the foregoing discussion that a wide range of Gram negative bacteria can grow in the contents of the hen's egg. Moreover, the available evidence does not appear to support the widely held belief (e.g. Tissier, 1926; Florian & Trussell, 1957) that the majority of contaminants of rotten eggs are of faecal origin.

The technical difficulties associated with sampling the yolk and white under strictly aseptic conditions has lead to the approximation (Brooks & Taylor, 1955) that the

contents of 90% of newly laid eggs are free from micro-organisms. From fresh eggs Gram positive bacteria, apparently ovarian in origin (Harry, 1963*a*), have been recovered most frequently whereas organisms capable of growing in the contents of the egg are of rare occurrence (Hadley & Caldwell, 1916; Haines, 1938; Miller & Crawford, 1953). Thus it would appear that the common contaminants of rotten eggs are normally of extragenital origin. The observation (Stuart & McNally, 1943) that they do not occur on the shell until after oviposition suggests that the nesting materials are the main reservoir of these organisms. Their occurrence on such commonly used materials as hay, straw, sawdust, wood shavings, etc., has been reported by Harry (1963*b*). He also found them on old droppings, but not on freshly voided faeces.

The microflora of the shells of commercial eggs is dominated by Gram positive bacteria (Haines, 1938; Board *et al.*, 1964) but only the Gram negative bacteria are capable of colonizing the contents of the egg (Board *et al.*, 1964) and this reflects presumably the selective nature of the antimicrobial defence of the albumen. The chelating agent, conalbumin, appears to be the most important component of this defence (Brooks, 1960; Garibaldi, 1960; Garibaldi & Bayne, 1960, 1962*a*,*b*; Board, 1964) and it has been demonstrated (Fraenkel-Conrat & Feeney, 1950; Feeney & Nagy, 1952) that Gram negative are less sensitive than Gram positive bacteria to the bacteriostatic action of this substance. It would appear from the evidence presented at the beginning of this discussion that the selectivity of this defence is not influenced by geographical factors, egg marketing procedures or the changing practices of poultry husbandry.

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References

Alford, L. R., Holmes, N. E., Scott, W. J. & Vickery, J. R. (1950). Studies in the preservation of shell eggs. 1. Nature of wastage in Australian export eggs. *Aust. J. appl. Sci.* **1**, 208.

Barritt, M. M. (1936). The intensification of the Voges Proskauer reaction by the addition of α-napthol. *J. Path. Bact.* **42**, 441.

Benjamin, E. W. (1914). The interior quality of market eggs. *Bull. Cornell agric. exp. Sta.* 353.

Bennetts, H. W. (1931). Specific putrefaction of hen eggs due to *Serratia* sp. *Aust. vet. J.* **7**, 27.

Bergey's Manual of Determinative Bacteriology, 6th ed. (1948). Edited by R. S. Breed, E. G. D. Murray and A. P. Hitchins. London: Ballière, Tindall & Cox.

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957). Edited by R. S. Breed, E. G. D. Murray and N. R. Smith. London: Ballière, Tindall & Cox.

Board, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.* **27**, 350.

Board, R. G. (1965). Bacterial growth on and penetration of the shell membranes of the hen's egg. *J. appl. Bact.* **28**, 197.

Board, R. G., Ayres, J. C., Kraft, A. A. & Forsythe, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.* **43**, 584.

Board, R. G. & Ayres, J. C. (1965). Influence of temperature on the course of bacterial infection of the hen's egg. *Appl. Microbiol.* **13**, 358.

Board, R. G. & Holding, A. J. (1960). The utilization of glucose by aerobic Gram-negative bacteria. *J. appl. Bact.* **23**, xi.

Brooks, J. (1960). Mechanism of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.* **23**, 499.

Brooks, J. & Taylor, D. J. (1955). Eggs and Egg Products. *Rep. Food Invest. Bd, Lond.*, no. 60. London: H.M.S.O.

Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bact.* **52**, 461.

Eddy, B. P. (1960). Cephalotrichous, fermentative Gram-negative bacteria: the genus *Aeromonas*. *J. appl. Bact.* **23**, 216.

Eddy, B. P. (1962). Further studies on *Aeromonas*. 1. Additional strains and supplementary biochemical tests. *J. appl. Bact.* **25**, 137.

Feeney, R. E. & Nagy, D. A. (1952). The antibacterial activity of the egg white protein conalbumin. *J. Bact.* **64**, 629.

Florian, M. L. E. & Trussell, P. C. (1957). Bacterial spoilage of shell eggs—IV. Identification of spoilage organisms. *Food Tech., Champaign* **11**, 56.

Fraenkel-Conrat, H. & Feeney, R. E. (1950). The metal-binding activity of conalbumin. *Arch. Biochem.* **29**, 101.

Garibaldi, J. A. (1960). Factors in egg white which control growth of bacteria. *Food Res.* **25**, 337.

Garibaldi, J. A. & Bayne, H. G. (1960). The effect of iron on pseudomonas spoilage of experimentally infected eggs. *Poult. Sci.* **39**, 1517.

Garibaldi, J. A. & Bayne, H. G. (1962*a*). Iron and bacterial spoilage of shell eggs. *J. Food Sci.* **27**, 57.

Garibaldi, J. A. & Bayne, H. G. (1962*b*). The effect of iron on *Pseudomonas* spoilage of farm washed eggs. *Poult. Sci.* **41**, 850.

Hadley, P. B. & Caldwell, D. W. (1916). The bacterial infection of fresh eggs. *Agric. exp. Sta. Rhode Island College*, Bull. no. 164.

Haines, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.* **38**, 338.

Haines, R. B. (1939). Microbiology in the preservation of the hen's egg. *Rep. Food Invest. Bd, Lond.* no. 47. London: H.M.S.O.

Haines, R. B. & Moran, T. (1940). Porosity of, and bacterial penetration through, the shell membrane of the hen's egg. *J. Hyg., Camb.* **40**, 453.

Harry, E. G. (1963*a*). Some observations on the bacterial content of the ovary and oviduct of the fowl. *Brit. Poult. Sci.* **4**, 63.

Harry, E. G. (1963*b*). The relationship between egg spoilage and the environment of the egg when laid. *Brit. Poult. Sci.* **4**, 91.

Henriksen, S. D. & Closs, K. (1938). The production of phenylpyruvic acid by bacteria. *Acta path. microbiol. scand.* **15**, 101.

Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.

Hugh, R. & Ryschenkow, E. (1960). An Alcaligenes-like *Pseudomonas* species. *Bact. Proc.* p. 78.

Hugh, R. & Ryschenkow, E. (1961). *Pseudomonas maltophilia*, an Alcaligenes-like species. *J. gen. Microbiol.* **26**, 123.

Hussong, R. V., Long, H. P. & Hammer, B. W. (1937). Classification of the organisms important in dairy products. II. *Pseudomonas fragi*. *Iowa Sta. Coll. Agric. exp. Sta. Res. Bull.* no. 225.

Johns, C. K. & Bérard, H. L. (1945). Further bacteriological studies relating to egg drying. *Sci. Agric.* **24**, 551.

Johns, C. K. & Bérard, H. L. (1946). Effect of certain methods of handling upon the bacterial content of dirty eggs. *Sci. Agric.* **26**, 11.

Kühr, C. A. H. von Wolzogen (1932). Uber eine Goringsmikrobe in Fakalien von Muckenlarven. *Zbl. Bakt.* (Abt. 2) **85**, 223.

Lagrange, E. (1935). Sur la flore des oeufs pourris. *Compt. rend.* **120**, 846.

Levine, M. & Anderson, D. Q. (1932). Two new species of bacteria causing mustiness in eggs. *J. Bact.* **27**, 337.

Manual of Microbiological Methods (1957). Committee on Bacteriological Technic, Society of American Bacteriologists. London: McGraw-Hill.

Miles, A. A. & Halnan, E. T. (1937). A new species of microorganism (*Proteus melanovogenes*) causing black rot in eggs. *J. Hyg., Camb.* **37**, 79.

Miles, E. M. & Miles, A. A. (1951). The identity of *Proteus hydrophilus* Bergey *et al.* and *Proteus melanovogenes* Miles and Halnan, and their relation to the genus *Aeromonas* Kluyver and van Niel. *J. gen. Microbiol.* **5**, 298.

Miller, W. A. & Crawford, L. B. (1953). Some factors influencing bacterial penetration of eggs. *Poult. Sci.* **32**, 303.

Paton, A. M. (1959). Enhancement of pigment production by *Pseudomonas*. *Nature, Lond.* **184**, 1254.

Pavarino, G. L. (1929). Changes produced in eggs by microorganisms. *Ann. Chim. Appl., Roma* **19**, 226.

Pennington, M. E., Jenkins, M. K., St. John, E. Q. & Hicks, W. B. (1914). A bacteriological and chemical study of commercial eggs in the producing areas of the middle west. *U.S. Dept. Agric. Bull.* no. 51.

Platt, A. E. & Anderson, C. F. (1939). Green rot in eggs. *J. Dept. Agric., S. Aust.* **42**, 1040.

Report (1958). Enterobacteriaceae Subcommittee. Report of Enterobacteriaceae Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies. *Int. Bull. bact. Nomen. Taxon.* **8**, 17.

Rhodes, M. E. (1958). The cytology of *Pseudomonas* spp. as revealed by a silver plating staining method. *J gen. Microbiol.* **18**, 639.

Rhodes, M. E. (1959). The characterization of *Pseudomonas fluorescens*. *J. gen. Microbiol.* **21**, 221.

Richard, O. & Mohler, A. (1950). Die Mikrobiologie der "Heu-eier", *Mitt. Lebensm. Hyg., Bern* **41**, 168.

Roessler, W. G. & McClung, L. S. (1943). Suggested method for use of vanillin as a test reagent for indole and skatole production by bacteria. *J. Bact.* **45**, 413.

Shimwell, J. L., Carr, J. G. & Rhodes, M. E. (1960). Differentiation of *Acetomonas* and *Pseudomonas*. *J. gen. Microbiol.* **23**, 283.

Spanswick, M. P. (1932). The cause of mustiness in eggs. *Amer. J. Publ. Hlth* **20**, 73.

Stewart, D. J. (1959). The phenylalanine test on Kligler's iron agar for the identification of *Proteus*. *Nature, Lond.* **183**, 1537.

Stuart, L. S. & McNally, E. H. (1943). Bacteriological studies on the egg shell. *U.S. egg poult. Mag.* **49**, 28.

Szybalski, W. (1950). A comparative study of bacteria causing mustiness in eggs. *Nature, Lond.* **165**, 733.

Thornley, M. J. (1960). The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *J. appl. Bact.* **23**, 37.

Tissier, H. (1926). Microbes de la putrefaction des oeufs. *Compt. rend.* **94**, 446.

Turner, A. W. (1927). *Achromobacter perolens* (h. sp.), the cause of mustiness in eggs. *Aust. J. exp. Biol. med. Sci.* **4**, 57.

Wrinkle, C., Weisser, H. H. & Winter, A. R. (1950). Bacterial flora of frozen egg products. *Food Res.* **15**, 91.

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A MICROBIOLOGICAL SURVEY OF THE INCUBATED EGGS OF CHICKENS AND WATERFOWL

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SYNOPSIS

Coliform organisms were the principal contaminants of rotten eggs from commercial duck hatcheries and incubated eggs of 45 species of waterfowl. Micrococci were the main contaminants of " incubator clears " obtained from a chicken hatchery. *Pseudomonas*, *Acinetobacter* and *Bacillus* spp were minor contaminants of the eggs of waterfowl. All the coliforms recovered from waterfowl eggs formed a large, well-defined capsule when grown on nutrient agar containing glucose or lactose.

INTRODUCTION

Eggs which have become rotten during storage have been the subject of many investigations and it has been established that Gram-negative bacteria are the principal contaminants (Board, 1965, 1968). Incubated eggs appear to have attracted little attention apart from those studies (Junghers, 1934; Davis, 1938; Harry, 1957; Pathak, Singh and Tangri, 1960) which have emphasised the rôle of coliform organisms in omphalitis and " non-specific " infections of young chicks. The present study was undertaken with the object of identifying the bacteria which occur in the contents of incubated eggs.

MATERIALS AND METHODS

The eggs

These were obtained from two commercial duck farms, a chicken hatchery and the Wildfowl Trust, Slimbridge, Gloucestershire. They had been removed from incubators or from under brooding hens because of rotting or failure to hatch. In the laboratory, the shells were swabbed with alcohol and pierced with a flamed scalpel. A sterile 4 mm inoculating loop or Pasteur pipette was used to transfer a little of the contents to dried plates of Violet Red Bile Agar (Oxoid Ltd) and Plate Count Agar (Oxoid Ltd).

Isolation of organisms

A conventional streaking method was used to spread the egg material over the surfaces of the above-noted media. After incubation (Violet Red Bile agar, 37 °C; Plate Count, agar 30 °C) only those plates containing more than about 50 colonies

were selected for further study. Representatives of each colony type were replated until purity was assured and the isolates were stored on slopes of Nutrient Agar (Oxoid Ltd) at 4 °C.

Characterisation of isolates

The methods summarised in Table 1 have been described in detail elsewhere (Board, 1965; Board and Board, 1968).

TABLE 1

A summary of properties used to identify bacteria from eggs

Organism	Properties: Morphology	Gram stain	Flagella	Catalase	Oxidase*	Action on glucose**	Remarks
Acinetobacter	CB	V	–	+	–	–	In routine examinations, the retention of traces of Crystal Violet by Gram-stained cells together with failure to produce acid in Hugh and Leifson's (1953) medium were found to be diagnostic properties.
Pseudomonas	R	N	Pol	+	+	O	The production of phenazine on medium of King, Ward and Raney (1954) together with growth at 41 °C were used for the identification of *Pseudomonas aeruginosa* (Stanier, Palleroni and Doudoroff, 1966).
Coliforms	R	N	NT	+	–	F	The following properties were used to identify *Escherichia coli*: Fermentation of lactose at 44 °C; Methyl Red test, positive; Voges Proskauer test, negative; Indole test, positive, and no growth on Simmons citrate agar (Edwards and Ewing, 1962).
Staphylococcus	C	P	NT	+	–	F	These organisms were characterised by the methods of Baird-Parker (1966). A positive phosphatase test and anaerobic utilisation of mannitol was used in the presumptive identification of *Staph. aureus*.
Micrococcus	C	P	NT	+	–	O	Subgroups of these organisms were characterised by the methods of Baird-Parker (1966).
Bacillus	R/S	P	NT	+	–	d	The form of colony and presence of endospore was used routinely for identification of isolates.
Streptococcus	C	P	NT	–	–	F	A small colony on Plate Count Agar together with a negative catalase reaction were used routinely for identification of these organisms.

CB, coccobacillus; C, cocci; R, rods; R/S, rods with endospores; V, partial retention of Crystal Violet in Gram-stained cells; N, negative; P, positive; Pol, polar, demonstrated by Rhodes' (1958) method; NT, not tested; +, positive reaction, * the method of Kovacs (1956), ** Hugh and Leifson (1953). O, oxidative; F, fermentative and d, different reactions.

Cytology

The dry method of Cruickshank (1965) was used to demonstrate capsules. The cells and nigrosine were smeared on a microscope slide, dried, fixed and stained with Crystal Violet.

RESULTS

Survey of waterfowl eggs

The majority of the eggs of waterfowl were addled and it was not possible to determine whether or not germinal development had occurred. " Dead-in-shells " were not examined. Three lots of eggs (40 eggs) were obtained from a commercial hatchery in the winter of 1969 and a further lot (55 eggs) from another hatchery in

TABLE 2

The species[1] of waterfowl whose eggs were included in this survey

Sub-family	**Anseranatinae**
Tribe	Anserantini
	Anseranas semipalmata (4)[2]
Sub-family	**Anserinae**
Tribe	Dendrocygnini (Whistling ducks, etc)
	Dendrocygna arborea (2); *Dendrocygna viduata* (5); *Dendrocygna bicolor* (12)
Tribe	Anserini (Swans and geese)
	Cygnus melanocoryphus (2); *Cygnus columbianus bewickii* (1); *Anser coerulescens coerulescens* (4); *Anser anser* (5); *Anser brachyrhynchus* (8); *Anser albifrons albifrons* (5); *Anser albifrons flavirostris* (3); *Anser fabalis* (1); *Anser indicus* (6); *Anser canagicus* (3); *Branta canadensis taverneri* (3); *Branta canadensis minima* (1); *Branta leucopsis* (2); *Branta canadensii moffitti* (2); *Branta canadensis occidentalis* (1)
Sub-family	**Anatinae**
Tribe	Tadornini (Shelducks and shellgeese)
	Tadorna variegata (5); *Chloëphaga melanoptera* (1); *Cereopsis novoe-hollandioe* (3); *Lophonetta specularioides specularioides* (4)
Tribe	Anatini (Dabbling ducks)
	Marmaronetta (Anas) angustirostris (4); *Anas georgica spinicauda* (3); *Anas bahamensis* (1); *Anas punctata* (1); *Anas aucklandica chlorotis* (5); *Anas luzonica* (5); *Anas superciliosa superciliosa* (2); *Anas laysanensis* (5); *Anas platyrhynchos platyrhynchos* (5); *Anas undulata* (7); *Anas wyvilliana* (3); *Anas falcata* (4); *Anas sibilatrix* (5); Others 3
Tribe	Somateriini (Eiders)
	Somateria mollissima foeroeensis (1); Others (5)
Tribe	Aythyini
	Netta rufina (1); *Aythya americana* (2); *Aythya australis australis* (2); *Aythya affinis* (4); *Aythya novoe-seelandioe* (5); *Aythya nyroca* (3)
Tribe	Cairinini (Wood ducks, etc)
	Aix spp (39), *Cairina moschata* (4); *Mergus* spp (3)
Tribe	Oxyurini
	Oxyura spp (5)
	Unidentified (5)

[1] The eggs from 45 species included in the survey: the nomenclature is that of Scott (1968).
[2] Number of eggs of species noted; total number of eggs examined, 200.

the summer of 1970. Seven batches of eggs (200 eggs) from the Wildfowl Trust were examined in the spring and summer of 1969 and a few eggs from the same source in the summer of 1970. The species of waterfowl included in the survey are given in Table 2. The identity of the bacteria isolated from the waterfowl eggs are given in Tables 4 and 5. It will be seen that coliforms accounted for 64% of isolates and

that, within this group, the majority (90%) were identified as *Escherichia coli* (lactose fermented at 44 °C; Methyl Red, positive; Indole, positive; Voges Proskauer, negative, and no growth on Simmons citrate agar).

Micrococci (Table 3) formed the next largest group of contaminants. Of 73

TABLE 3

Identity[1] *of organisms recovered from the eggs of waterfowl*

	Domestic duck				
	Hatchery A	Hatchery B	Waterfowl[2]	Number	Total percentage of isolates
Number eggs examined	40	55	200	...	...
Coliforms	53	10	140	203	64
Staphylococcus	2	1	24	27	8·5
Micrococcus	1	0	45	46	15
Pseudomonas spp	13	9	0	22	7
Pseudomonas aeruginosa	0	5	0	5	2
Acinetobacter	5	0	0	5	2
Bacillus spp	7	0	2	9	3
Number isolates	81	25	211	317	...

[1] A representative of each colony type was taken from every plate.
[2] Species given in Table 2.

isolates, 27 were identified with *Staphylococcus* (they were not examined for coagulase production) and 46 with *Micrococcus* as defined by Baird-Parker (1966). The majority of these organisms were isolated from the eggs of wild waterfowl and the incidence of their recovery increased during the survey. Thus relatively few isolates were obtained in spring and the majority were recovered in the summer, a trend that was contrary to that noted for infection of eggs with moulds. In eggs which transmitted light, brown or red spots or patches of mycelia with dark edges were seen on the underside of the shell. In broken out eggs, patches of mould in the shell membranes were overlaid with gelatinised albumen. It is tempting to suggest that these contrasting patterns of recovery were associated with the humidity of the birds' environment, the growth of moulds being favoured by the wet spring and the survival of the micrococci by the dry summer.

Pseudomonads and acinetobacters (Table 3) were minor (about 9% of isolates) contaminants and they were recovered only from the eggs of domestic ducks, in which they were found together with coliforms. The latter, however, were not isolated from eggs which contained *Pseudomonas aeruginosa*. Another minor group, the *Bacillus* spp, tended also to be the sole contaminant in the eggs from which they were recovered. The bacilli formed copious slime when grown on Plate Count Agar.

The hen's egg

A different picture was obtained from the survey of eggs (1,160) obtained from a chicken hatchery (Tables 4 and 5). The eggs were " clears ", germinal development had not occurred and none of the eggs was opaque to transmitted light. Thirteen per cent of the eggs had cloudy whites and most of these had custard-like material on the surface of the yolk. Twenty five per cent of the eggs harboured

TABLE 4

Incidence of contamination[1] of contents of albumen of incubator rejects obtained from a chick hatchery

Lot number	Number of eggs	Eggs containing viable organisms in albumen		Eggs having cloudy white		Organisms recovered[2]	
		Number	Percentage	Number	Percentage	Gram +ve	Gram −ve
1	180	38	21	35	19	34	6
2	160	61	38	28	17·5	62	3
3	160	63	39	51	32	79	2
4	120	44	28	21	17·5	32	0
5	180	24	13	8	4·5	35	2
6	180	46	25	1	7[1]	45	6
7	180	43	24	9	5	37	7
Totals	1,160	209	25	153	13	324	26

[1] A 4 mm loop of albumen spread over Plate Count Agar; contents only considered contaminated when 50 colonies developed.

[2] Representatives of all colony types on Plate Count Agar were stained by Gram's method and sub-cultured for further characterisation.

TABLE 5

The identity of organisms isolated from incubator rejects obtained from a commercial chicken hatchery

Organism	Subgroup or species	Number of strains examined	Total
Staphylococcus[1]	I[2] (*aureus*)	18	
	II	4	
	III	42	74
	IV	5	
	V	5	
Micrococcus[1]	1	2	
	2	5	
	3	3	
	4	1	205
	5	51	
	6	141	
	7	2	
Streptococcus	Not identified further	4	4
Coliforms	*Escherichia coli*	25	
	Aerobacter aerogenes	2	38
	Unclassified	11	
			321

[1] Identified with the scheme of Baird-Parker (1966).

[2] Coagulase positive.

large numbers of organisms in their contents. In contrast to the eggs of waterfowl, micrococci were the dominant contaminants of the hen's eggs. Of the 350 bacteria isolated in pure culture, 324 were Gram-positive cocci and, of the 321 characterised in detail, 74 were identified with *Staphylococcus*, 205 with *Micrococcus*, 4 with *Streptococcus* and 36 as coliforms. Of the latter, 25 were identified with *E. coli* (as defined above).

Capsule formation

The colonies formed on Violet Red Bile Agar by coliforms derived from the eggs of waterfowl were large and mucoidy whereas those from the hen's eggs formed small colonies with a matt surface. The cells present in the mucoidy colony were enclosed in large, well-formed capsules. Attempts to demonstrate capsules on cells present in rotten eggs were unsuccessful because of the failure of the staining techniques with this material. It was noted, moreover, that the coliforms obtained from waterfowl eggs did not form capsules unless glucose or lactose (final concentration, 0·5% w/v) was added to Nutrient Agar.

DISCUSSION

This study has shown that, like the rots recovered from hen's eggs which had been intended for human consumption (Board, 1965), Gram-negative bacteria were the principal contaminants of the rotten (incubated) eggs of waterfowl. There were, however, marked differences in the proportions of species of bacteria recovered from these two sources of eggs. Coliforms, the majority of which were identified with *E. coli*, were the predominant contaminants of duck eggs whereas *Escherichia*, *Hafnia* and *Aerobacter* spp were minor contaminants of the rotten eggs of hens (Board and Board, 1968). In the latter eggs, *Pseudomonas*, *Acinetobacter* and *Alcaligenes* spp have been frequently isolated (Board, 1965) but, in the present study, only a few strains were recovered from the eggs of waterfowl (Table 3).

When discussing factors contributing to the dominance of Gram-negative bacteria in rotten eggs, Board (1968) surmised that these organisms were selected because of their relatively simple nutritional requirements and ability to grow, albeit slowly (Feeney and Nagy, 1952), in the presence of the iron chelating agent, conalbumin (ovotransferrin). Subsequently it has been demonstrated (Seviour and Board, 1972) that the selection occurs whilst the micro-organisms are retained in the shell membrane and that temperature is an important, extrinsic factor in the process. Thus pseudomonads and related organisms became dominant in eggs held at room temperature or less whereas coliforms did so in eggs held at 37 °C. In the present study it was noted that the shells of all the eggs were badly stained. Thus, poor hygiene in the nesting areas had provided ample opportunity for the shells to acquire organisms of faecal origin and it would seem reasonable to deduce that the coliforms had been preferentially selected when the eggs were incubated.

The results obtained from a microbiological survey of the incubated eggs of the hen contrasts with those discussed above and at this time it is not possible to account for the dominance of the micrococci. It is known that they can be the predominant contaminants on the shells of clean eggs in the nest (Haines, 1938; Board, Ayres, Kraft and Forsythe, 1964)—the eggs included in this survey were apparently naturally clean—but that, when translocated to the underlying membranes, they are overgrown by Gram-negative bacteria (Seviour and Board, 1972).

It was noted that pure cultures of some of the micrococci isolated in the present study formed large populations when placed on the inner membrane of the air space of eggs maintained at 37 ° to 40 °C. Thus it is possible that the contaminants recovered from the incubated eggs of hens could have been derived from the shell and that some feature such as the production of eggs in dusty atmospheres or

selection by fumigation had led to the shell membranes becoming contaminated with micrococci alone. Alternatively, they may have originated from the oviduct, a possibility suggested by the observations of Harry (1963*a*, *b*). Previous investigators (Harry, 1957; Fuller and Jayne-Williams, 1968, 1970) have recovered micrococci from the yolk sac and organs of newly hatched chickens but, apart from infections with *Staphylococcus aureus* (Harry, 1957), these organisms do not appear to be pathogenic at this stage in the chicks' development. It would be unwise, however, to consider that the micrococci are harmless contaminants of the contents of incubating eggs until it has been demonstrated that they have no adverse influence on the early stages of the embryo's development.

Although many studies (Junghers, 1934; Davis, 1938; Harry, 1957; Pathak, Singh and Tangri, 1960) have shown that coliforms are important casual agents of yolk sac and " non-specific " infections of young chicks, no one has noted encapsulation of the organisms isolated in pure culture. It will be recalled that this was a feature of the coliforms which we recovered from the eggs of a large number of species of waterfowl. As capsule formation in an inherited property of the coliform organisms, it would seem that some property of duck eggs is selective for such organisms and we have noted that encapsulation is enhanced when our isolates are passaged in the albumen of duck eggs. It would be of interest to know if this property of the albumen of waterfowl is selective for smooth rather than rough strains of *Enterobacteriaceae* in general and if such a selective process is partly responsible for the poor reputation which duck eggs have with persons who are concerned with public health.

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REFERENCES

Baird-Parker, A. C. (1966). Methods for classifying staphylococci and micrococci. In *Identification Methods for Microbiologists*, p. 59. Edit. Gibbs, B. M. and Skinner, F. A. London, Academic Press.

Board, P. A. and Board, R. G. (1968). A diagnostic key for identifying organisms recovered from rotten eggs. *Br. Poult. Sci.*, **9**: 111-120.

Board, R. G. (1965). The properties and classification of the predominant bacteria in rotten eggs. *J. appl. Bact.*, **28**: 437-453.

Board, R. G. (1968). Microbiology of the egg: a review. In: *Egg Quality: A Study of the Hen's Egg*, pp. 133-162. Edit. Carter, T. C. Edinburgh, Oliver and Boyd.

Board, R. G., Ayres, J. C., Kraft, A. A. and Forsythe, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.*, **43**: 584-595.

Cruickshank, R. (1965). *Medical microbiology*. Edinburgh, Livingstone.

Davis, C. R. (1938). Colibacillosis in young chicks. *J. Am. vet. med. Sci.*, **92**: 518-522.

Edwards, P. R. and Ewing, W. H. (1962). *Identification of Enterobacteriaceae*, 2nd ed. Minneapolis, Burgess Publishing Co.

Feeney, R. E. and Nagy, D. A. (1952). The antimicrobial activity of the egg white protein conalbumin. *J. Bact.*, **64**: 629-643.

FULLER, R. AND JAYNE-WILLIAMS, D. J. (1968). The origin of bacteria recovered from the peritoneum and yolk sac of healthy chickens. *Br. Poult. Sci.*, **9**: 159-163.

FULLER, R. AND JAYNE-WILLIAMS, D. J. (1970). Resistance of the fowl (*Gallus domesticus*) to invasion by its intestinal flora. I. The effect of hormonal bursectomy on the invasiveness of the intestinal microflora of the fowl. *Res. vet. Sci.*, **11**: 363-367.

HAINES, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.*, **38**: 338-355.

HARRY, E. G. (1957). The effect on embryonic and chick mortality of yolk contamination with bacteria from the hen. *Vet. Rec.*, **69**: 1433-1441.

HARRY, E. G. (1963*a*). Some observations on the bacterial content of the ovary and oviduct of the fowl. *Br. Poult. Sci.*, **4**: 63-90.

HARRY, E. G. (1963*b*). The relationship between egg spoilage and the environment of the egg when laid. *Br. Poult. Sci.*, **4**: 91-100.

HUGH, R. AND LEIFSON, E. (1953). The taxonomic significance of fermentation versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.*, **66**: 24-26.

JUNGHERS, E. (1934). Diseases of brooder chicks. *Bull. Conn. agric. Exp. Stn*, **202**.

KING, E. D., WARD, M. K. AND RANEY, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. clin. Med.*, **44**: 301-307.

KOVACS, M. (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature, Lond.*, **178**: 703-704.

PATHAK, R. C., SINGH, C. H. AND TANGRI, R. P. (1960). Chick mortality and the contamination of yolks by members of the Enterobacteriaceae. *Br. vet. J.*, **116**: 81-84.

RHODES, M. E. (1958). The cytology of *Pseudomonas* spp as revealed by a silver plating staining method. *J. gen. Microbiol.*, **18**: 639-648.

SCOTT, P. (1968). *A Coloured Key to the Wildfowl of the World*. London, Royle.

SEVIOUR, E. M. AND BOARD, R. G. (1972). The behaviour of mixed bacterial infections in the shell membranes of the hen's egg. *Br. Poult. Sci.*, **13**: 33-43.

STANIER, R. Y., PALLERONI, M. J. AND DOUDOROFF, M. (1966). The aerobic pseudomonads; a taxonomic study. *J. gen. Microbiol.*, **43**: 159-271.

A DIAGNOSTIC KEY FOR IDENTIFYING ORGANISMS RECOVERED FROM ROTTEN EGGS

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SYNOPSIS

A collection of 119 cultures of bacteria was assembled from eggs which had rotted on the premises of producers. All but 13 of the isolates were Gram-negative. These were characterised in detail and the data used (*i*) to devise a determinative key that permits rot-producing bacteria to be identified at generic level, and (*ii*) to establish systems whereby identification at species level can be achieved with the minimum of tests. With the use of these schemes the following were recognised: *Pseudomonas fluorescens*, *Pseudomonas maltophilia*, *Aeromonas liquefaciens*, and members of *Flavobacterium*, *Cytophaga*, *Proteus*, *Escherichia*, *Hafnia* and *Aerobacter*. The literature dealing with the microbiology of rotten eggs was reviewed with the object of assessing the incidence of the organisms listed above in rotten eggs of commercial origin.

INTRODUCTION

The microbiology of rotten eggs has been the subject of many investigations (Pennington, Jenkins, St John and Hicks, 1914; Tissier, 1926; Pavarino, 1929; Bennetts, 1931; Lagrange, 1935; Miles and Halnan, 1937; Haines, 1938; Alford, Holmes, Scott and Vickery, 1950; Miller and Crawford, 1953; Florian and Trussell, 1957). A notable feature of all these studies was the emphasis given to characterisation of organisms with the sole objective of identifying them with definitions of species given in treatises concerned with determinative bacteriology. Thus from the standpoint of systematic bacteriology, much is known about the types of organisms that are of common occurrence in rotten eggs (Board, 1965). However, the concern with the niceties of bacterial taxonomy, particularly nomenclature, has produced a body of information that would appear to be of limited value to poultry scientists and technologists. There is a clear need for (1) a scheme whereby these persons, using the minimum number of tests, can identify organisms recovered from addled eggs and (2) information that would permit them to determine whether or not their isolates were common or uncommon contaminants of eggs. It was with these objectives in mind that the present study was undertaken. A collection of bacteria was assembled from eggs that had rotted on the premises of the producer. These were characterised in detail. Descriptions are given of those methods which allowed the rot-producing bacteria to be characterised sufficiently for identification at specific or, occasionally, generic level.

MATERIALS AND METHODS

Isolation of organisms. Rotten eggs were obtained during the autumn of 1966

from a local egg packing station. These had rotted on the premises of the producer and were rejected during candling immediately on receipt at the station. In the laboratory, the eggs were examined by methods described previously (Board, 1965). Briefly, the shells were wiped with alcohol, flamed and then pierced with a sterile scalpel. The salient features of the rot were noted and a loopful of the contents streaked on plate-count agar (Oxoid Ltd, London). The plates were examined after 3 and 5 days' incubation at 27° C. and representatives of each type of colony were transferred to plate-count agar. The purity of the isolates was ensured by replating on at least three occasions. They were maintained on slopes of nutrient agar (Oxoid) at 4° C. and sub-cultured at intervals of 6 months at which time, also, their purity was checked.

Characterisation of isolates. An 18-hr culture in nutrient broth (Oxoid) was used as the inoculum in all experiments and all tests were made at 27° C. except in the case of nutrient gelatin (Oxoid) which was incubated at 22° C.

Fermentation tests. The medium of Hugh and Leifson (1953) was used. The fermentation substrate, which was sterilized by Seitz filtration, was added to liquefied medium cooled to 45° C. to give a final concentration of 0·5 per cent (w/v).

Acetoin production and MR test. Cultures in the MR-VP medium (Oxoid) were examined by Barritt's (1936) method—*i.e.* 1 ml. of 40 per cent (w/v) KOH and 3 ml. of a 5 per cent (w/v) solution of α-naphthol in amyl alcohol were added to *c.* 2·5 ml. of medium. Acetoin production was indicated by the development of a strong red colour. The *p*H of the culture was tested with methyl red.

Starch hydrolysis. Plates of nutrient agar containing 1·0 per cent (w/v) of soluble starch were inoculated on the surface and after incubation flooded with a solution of Lugol's iodine. Hydrolysis was indicated by clear zones.

H_2S production. The butt at the base of slopes of triple sugar iron agar (Oxoid) was stab inoculated: a blackening of the medium was taken as evidence of the liberation of H_2S.

Indole. Cultures in a 1 per cent (w/v) aqueous solution of tryptone (Oxoid) were tested with Kovac's reagent—10 g. *p*-dimethyl-aminobenzaldehyde dissolved in 150 ml. of amyl alcohol followed by acidification with concentrated HCl (50 ml.). Indole production was indicated by the development of a red coloration.

Arginine hydrolysis test. Medium 2A of Thornley (1960) was dispensed in 3 ml. amounts in 5 ml. screw-capped bottles. After inoculation the remaining free air space was filled with sterile mineral oil. Hydrolysis of arginine was indicated by the development of an alkaline reaction.

Urea hydrolysis. Those bacteria which produced an alkaline reaction within 18-24 hr in the medium of Christensen (1946) were recorded as urease positive.

Denitrification. The medium of Stanier, Palleroni and Doudoroff (1966) was used. The development of a marked opacity with or without gas production was taken to be evidence of denitrification.

Oxidase test. A loopful of cells from a nutrient agar culture was placed on filter paper moistened with 1 per cent (w/v) aqueous solution of *p*-amino-N:N-dimethyl aniline (laboratory reagent, B.D.H. Ltd). The development of a blue colour within 30 sec. was recorded as a positive reaction.

Pigment production in pseudomonads. Organisms were streaked on slopes of medium A and medium B of King, Ward and Raney (1954). Medium A favours

the production of phenazine (a blue, water-soluble, chloroform-insoluble pigment) though some pseudomonads produce a slight amount of fluorescent pigment on it. Medium B, which suppresses the production of phenazine, encourages the production of fluorescent pigments—water-soluble yellowish to bluish green pigments which fluoresce strongly when exposed to ultraviolet irradiation.

Liquefaction of gelatin. Nutrient gelatin (Oxoid Ltd) was stabbed with a straight wire and incubated at 22° C.

Cytological observations. The Hucker modification of the Gram-staining procedure was used (Committee on Bacteriological Technic, 1957). When a stained film did not give a suitable preparation for the determination of the shape and arrangement of an organism, dried films on microscope slides were negatively stained by the method described by Cruickshank (1965).

RESULTS AND DISCUSSION

Preliminary separation of isolates

A collection of 119 isolates was assembled from the 84 eggs examined in this study. Of these eggs, 32 exhibited early signs of mould infection, the inner surface of the shell membranes supporting patches of mycelium embedded in gelatinised albumen. The contents of the eggs were, however, heavily contaminated with bacteria and the yolks and whites showed changes that are normally associated with bacterial action. Consequently the bacteria recovered from such eggs were included in this study.

Table 1 gives details of the tests which were of diagnostic value in the preliminary characterisation of the isolates. The data obtained from these tests were used to devise the determinative key set out in Table 2. The provisional grouping and presumptive identification of the isolates are summarised in Table 3. It will be noted that Gram-negative organisms were the dominant contaminants of the eggs. Moreover, the range of organisms recovered in this study was essentially the same as those of previous investigators (Haines, 1938; Florian and Trussell, 1957; Board, 1965). Thus it was concluded that the material used in this study was sufficiently extensive to include a normal and representative range of both bacterial types and egg rots.

All of the organisms isolated in this study were tested for their ability to produce macroscopic changes of the yolk and white of eggs. In practice the air cell of newly laid eggs were inoculated with about 0·5 ml. of a broth culture and the eggs, with air cells uppermost, held at room temperature for upwards of 6 weeks. They were candled frequently and when rotting was noted the shells were broken and the salient features of the rot recorded. The results are summarised in Table 3. Organisms that caused detectable changes in the yolk and white were characterised in detail and the results are considered hereunder.

Detailed characterisation of isolates

Fluorescent pseudomonads. These organisms were characterised by the methods discussed by Stanier *et al.* (1966) and features of diagnostic value for the differentiation of species are given in Table 4. It will be seen that two species were recovered in this study, *Pseudomonas fluorescens* and *Pseudomonas putida.*

TABLE 1

A summary of the tests used for the preliminary identification of organisms recovered from eggs

Medium	Observation
Nutrient agar—streaked to give isolated colonies	Appearance of colony Gram stain Morphology—checked by negative staining Oxidase test Catalase test
Nutrient agar containing 1·0 per cent (w/v) starch	Absence of blue coloration on addition of Lugol's iodine taken as evidence of starch hydrolysis
Christensen's (1946) urea medium	Development of alkaline reaction is taken as evidence of urea hydrolysis
Thornley's (1960) Arginine medium	Alkaline reaction evidence of arginine utilisation
Hugh and Leifson's (1953) medium containing 0·5 per cent (w/v) glucose	Breakdown of carbohydrate, *viz.* oxidative, acid formed in the presence of molecular oxygen; fermentative, acid formed both in presence and absence of molecular oxygen
Litmus milk	Change in *p*H Digestion Pigment production—checked under ultra-violet irradiation

TABLE 2

First-stage diagnostic table for organisms recovered from rotten eggs

Property	Fluorescent pseudomonads	*Pseudomonas maltophilia*	*Flavobacterium*	*Cytophaga*	Organism C	*Alcaligenes*/*Achromobacter*	*Aeromonas*	*Proteus*	*Enterobacter*	*Bacillus*	*Arthrobacter*	*Micrococcus*	*Streptococcus*
Reaction to Gram's stain	−	−	−	−	−	−	−	−	−	+	+	+	+
Morphology	R	R	R	R	R	R	R	R	R	R	C	Co	Co
Action on glucose	O	−	O	−	O	−	F	F	F	O/F	O	...	...
Hydrolysis of starch	−	−	−	−	−	−	+	−	−	d	...	...	...
Hydrolysis of urea	−	−	−	−	−	−	−	+	−	d	...	...	...
Hydrolysis of arginine	+	−	−	−	−	−	+	−	−	−	...	...	...
Litmus milk													
*p*H	K	−	K	K	K	d	d	K	d	d	...	...	...
digestion	+	+	d	+	−	d	d	+	d	d	...	...	...
odour	−	AC	−	−	−	d	d	−	d	d	...	...	...
clot—soft	−	+	−	−	−	d	d	−	d	d	...	...	...
hard	−	−	−	−	−	d	d	−	d	d	...	...	...
Colony													
Appearance-characteristic	−	−	−	−	CR	−	−	SW	−	−	...	...	...
spreading	−	−	−	+	−	−	−	−	−	−	...	...	...
swarming	−	−	−	−	−	−	−	+	−	−	...	...	...
pigment	BG	y	Y	Y	Y	−	−	−	−	−	...	...	...
Oxidase	+	−	+	+	+	d	+	−	−	−	...	...	...
Catalase	+	+	+	+	+	+	+	+	+	+	...	+	−

+, positive; −, negative or no change; AC, almond-like odour; BG, blue-green; C, Coryneform; Co, Coccus; CR, crumbly; d, different reactions among strains; F, fermentation; K, alkaline reaction; O, oxidation; R, rod; SW, growth swarms over moist nutrient agar; y, light yellow pigmentation; Y, marked yellow pigmentation.

TABLE 3

The incidence of different types of bacteria in the rotten eggs examined in this study

Classification	No. of isolates	Action on egg[1]
Fluorescent pseudomonad	22	Fluorescent green rot or pink rot
Pseudomonas maltophilia	3	Green rot
Flavobacterium	5	Yellow pigment formed in shell membranes
Cytophaga	1	Yellow pigment formed in shell membranes
Organism C	6	Brown discoloration of albumen
Alcaligenes/Achromobacter	35	None
Aeromonas	4	Black rot, type 1
Proteus	20	Black rot, type 2
Enterobacter	10	Custard rot or no change
Bacillus	3	None
Arthrobacter	4	None
Micrococcus	6	None
Streptococcus	...	None

[1] Details of changes given by Board (1965).

TABLE 4

Characters used for the differentiation of fluorescent pseudomonads

	Pseudomonas		
	aeruginosa[1]	*fluorescens*	*putida*
Pigments[2]: Fluorescent	+	+	+
Phenazine	+	d	−
Denitrification	+	d	−
Growth at: 4° C.	−	+	d
41° C.	+	−	−
Gelatin liquefaction	+	+	−
No. of isolates	0	14	8

+, positive reaction; −, negative reaction; d, different reactions among the strains studied.

[1] Although this species was not isolated, its properties are given for the convenience of the reader.

[2] Tested with the medium of King, Ward and Raney (1954).

Fluorescent pseudomonads have long been recognised as common contaminants of rotten eggs (Pennington *et al.*, 1914; Alford *et al.*, 1950; Florian and Trussell, 1957) whereas those organisms (*Pseudomonas aeruginosa*) that produce phenazine in addition to fluorescein have been recovered infrequently (Miles and Halnan, 1937; Platt and Anderson, 1939). With the first-mentioned organisms, numerous specific epithets were used by earlier investigators, *viz Ps. syncyanea* by Pennington *et al.* (1914) and *Bacillus fluorescens liquifaciens* by Pavarino (1929). This was noted by Haines (1938) but he was of the opinion that contemporary definitions of species were based on properties of doubtful taxonomic significance. Consequently he did not attempt specific identification of the fluorescent pseudomonads he had recovered from rotten eggs. Later workers (Florian and Trussell, 1957; Board, 1965) identified their isolates with *Pseudomonas fluorescens* as defined by Breed, Murray and Smith (1957) or by Rhodes (1959). The latter's definition attached no importance to an

organisms' action on gelatin yet evidence had been at hand for many years (Flügge, 1886; den Doreen de Jong, 1926) that the failure of a fluorescent pseudomonad to hydrolyse gelatin was but one of a number of properties that such organisms did not share with proteolytic types. This was confirmed by Stanier *et al.* (1966)—see Table 4; they reserved the specific epithet *fluorescens* for the protein-splitting strains and reaffirmed the value of using *putida* for the others. It is noteworthy that these two species produce fundamentally different changes in eggs. *Ps. fluorescens* causes the rot referred to as " pink rot " by Haines (1939) whereas *Ps. putida* produces the " fluorescent green rot " described by Board (1964).

Pseudomonas maltophilia. Of the rot-producing organisms recovered in this study, these proved to be the most difficult to recognise. The following features were useful: the negative oxidase test, the failure to change the colour of the acid-base indicator in Hugh and Leifson's (1953) medium containing glucose, or Thornley's (1960) medium containing arginine and the slow digestion of gelatin (incubation, 22° C.). No change in the *p*H was detected when these organisms grew in skim milk containing litmus or bromothymol blue: the milk was slowly digested and a soft clot formed at the bottom of the tube. In addition, an almond-like (nutty) odour was produced and, of all the organisms that the present authors have isolated from rotten eggs, this features appears to be peculiar to *Ps. maltophilia.*

These organisms appear to be infrequent contaminants of rotten eggs. A few strains were isolated by Florian and Trussell (1957) but these were identified as *Alcaligenes bookeri.* The bacteria referred to as pseudomonad E (Board, 1965) would now be identified as *Ps. maltophilia.* In the present study, only three strains were isolated.

Flavobacterium. The Gram-negative aerobic rods of this genus formed a round, raised to convex colony with an entire edge and of a butter-like consistency on nutrient agar. The colony was yellow but the pigment never diffused into the medium. All the isolates gave a positive oxidase test. They all failed to grow or change the *p*H in the arginine medium of Thornley (1960). Due to the confusion existing in the taxonomy of this group, identification at species level was not attempted.

Members of this genus have been recovered from the shell of eggs intended for human consumption (Haines, 1938), egg packaging materials (Board, Ayres, Kraft and Forsythe, 1964) and rotten eggs (Florian and Trussell, 1957). The present study appears to be the first in which they have been associated with changes in the appearance of the hen's egg (Table 3). When they were placed in the air cell of fresh eggs, a yellow pigment was formed in the inner shell membrane.

Cytophaga. The form of the colony was the feature which distinguished organisms of this genus from the other aerobic, Gram-negative rods. In the 24 hr following the streaking of nutrient agar, a yellow, raised to convex colony of irregular margin and butyrous consistency developed. Thereafter a thin film of growth spread progressively out from the initial colony. This had a yellow, mucoid appearance in reflected light and was irridescent with transmitted light. Such a colony form is typical of *Cytophaga*, a genus containing organisms with flexuous cell walls that " creep " over solid surfaces. These organisms have been recovered from the shell of eggs (Board *et al.*, 1964) but the present report appears to be the first in which they have been associated with changes in the appearance of the egg's contents.

With eggs infected in the laboratory, the shell membrane at the site of inoculation was stained with the pigment produced by the organism.

Organism C. This was recognised by the appearance of the colony developing on nutrient agar. It was yellow, wart-like in appearance and crumbly in texture. The cells were rods of regular shape; they were Gram-negative and non-acid fast. These properties were sufficient to allow this organism to be recognised when it was recovered along with others from a rotten egg. It has not been characterised in further detail and the available information does not allow identification even at generic level. The changes occurring in eggs inoculated in the laboratory was another feature peculiar to organism C. A deep brown discoloration was evident at the site of inoculation (the air cell membrane) and the pigment diffused slowly through the albumen. The present study would appear to be the first in which this organism has been recovered from rotten eggs.

Alcaligenes-Achromobacter. This was a heterogeneous collection of aerobic Gram-negative rods none of which produced detectable changes when inoculated in newly laid eggs. Consequently, they were not studied in detail.

Organisms of this type appear to be common contaminants of rotten eggs; they have been isolated by Miles and Halnan (1937), Alford *et al.* (1950), Richard and Mohler (1950), Florian and Trussell (1957) and Board (1965).

Aeromonads. These gram-negative organisms were easily distinguished from other fermentative organisms by virtue of their positive oxidase reaction, their ability to hydrolyse starch and to utilise arginine in the medium of Thornley (1960). Additional properties are given in Table 5. These organisms were identified as *Aeromonas liquefaciens* as defined by Eddy (1960, 1962).

TABLE 5

Characters of diagnostic value for identification of Aeromonas liquefaciens

Property	*Aeromonas liquefaciens*
Motility	+
Glucose—Acid	+
Gas	+
MR	±
VP	+
Indole	+
Starch hydrolysis	+
Arginine dihydrolase	+
No. of isolates	4

+, positive reaction; ±, some strains gave a weak positive.

When originally isolated from rotten eggs, these organisms were considered to be a new species to which the name *Proteus melanovogenes* was given (Miles and Halnan, 1937). Subsequent examinations of some of the original isolates resulted in their being assigned (Miles and Miles, 1951) to *Aeromonas* and identified (Eddy, 1960) as *A. liquefaciens*. Aeromonads appear to be infrequent contaminants of rotten eggs: 6 strains were isolated by Florian and Trussell (1957), 6 by Board (1965) and 3 in the present study.

Proteus. The rapid hydrolysis of urea and the swarming growth on nutrient agar were the distinguishing features of this group of fermentative Gram-negative rods. They were characterised further by the methods discussed by Edwards and

Ewing (1962). The results are given in Table 6 from which it will be seen that 4 species were recovered in this study, namely *Proteus vulgaris*, *Pr. morganii*, *Pr. mirabilis* and *Pr. rettgeri*. As far as can be ascertained the last 3 have not been isolated from rotten eggs in previous investigations.

TABLE 6

Characters of diagnostic value for differentiation of Proteus *spp.*

	Proteus			
	vulgaris	*mirabilis*	*morganii*	*rettgeri*
Glucose—Acid	+	+	+	+
Gas	+	+	+	+
Methyl red	+	+	+	+
Urease	+	+	+	+
Gelatin liquefaction	+	+	−	−
Sucrose	+	+	−	+
Indole	+	−	+	+
Citrate	−	+	−	+
No. of strains isolated	5	11	1	3

+, positive reaction; −, negative.

Many workers have asserted that black rots in eggs are caused by species of *Proteus* (Haines, 1938; Alford *et al.*, 1950; Florian and Trussell, 1957). The validity of these claims has been questioned (Board, 1965) because of the workers' failure to present evidence that allows a reader to separate *Proteus* from *Aeromonas* and other non-lactose-fermenting organisms. In consequence it is difficult to assess the importance of members of this genus as causative agents of rotting of eggs intended for human consumption or to evaluate the claims (Alford *et al.*, 1950; Frazier, 1967) that their growth in eggs is favoured by storage at 20° C. or above.

Enterobacters. This general term was applied to the group of Gram-negative rods that fermented glucose but failed to digest starch, hydrolyse urea or give a positive oxidase reaction. They were characterised further by the methods discussed by Edwards and Ewing (1962) and the results are set out in Table 7. It will be seen that species of *Escherichia*, *Hafnia* and *Aerobacter* were isolated in this study.

TABLE 7

Characters of diagnostic value for differentiation of enterobacters

	Escherichia	*Hafnia*	*Aerobacter*
Glucose—acid	+	+	+
Gas	+	+	+
Lactose	+	−	+
Methyl red	+	−	−
Voges Proskauer	−	+	+
Gelatin	−	−	+
Indole	+	−	−
Citrate	−	d	+
No. of isolates	1	5	5

+, positive reaction; −, negative reaction; d, different reactions among the strains studied.

Organisms with properties similar to the enterobacters obtained in this study have been recovered from rotten eggs by numerous investigators. Some (Tissier, 1926; Lagrange, 1935; Haines, 1938) regarded them as chance contaminants which did not contribute to the deterioration of eggs, while others (Pennington *et al.*, 1914; Richard and Mohler, 1950; Frazier, 1967) consider them to be causative agents of taints in eggs intended for human consumption. It is only recently (Florian and Trussell, 1957; Board, 1965) that macroscopic changes of the yolk and white have been attributed to certain of these organisms; the liquefaction of gelatin and breakdown of egg-yolk emulsion appear to be features peculiar to such organisms. It is noteworthy that enterobacters with proteolytic activity were recovered in the present study and that they caused rots in eggs infected in the laboratory (Table 3).

Gram-positive bacteria. It will be seen in Table 3 that a few types of Gram-positive bacteria were isolated. In keeping with general experience, these failed to change the appearance of the yolk or white when inoculated in fresh eggs. As they are known to be representative of the numerically predominant contaminants of the egg shell (Board *et al.*, 1964) it would appear reasonable to assume that they penetrated the shell together with the organisms that caused the egg to rot.

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REFERENCES

Alford, L. R., Holmes, N. E., Scott, W. J. and Vickery, J. R. (1950). Studies in the preservation of shell eggs. 1. Nature of wastage in Australian export eggs. *Aust. J. appl. Sci.*, **1**: 208-214.

Barritt, M. M. (1936). The intensification of the Voges Proskauer reaction by the addition of α-napthol. *J. Path. Bact.*, **42**: 441-454.

Bennetts, H. W. (1931). Specific putrefaction of hen eggs due to *Serratia* sp. *Aust. vet. J.*, **7**: 27-29.

Board, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.*, **27**: 350-364.

Board, R. G. (1965). The properties and classification of the predominant bacteria in rotten eggs. *J. appl. Bact.*, **28**: 437-453.

Board, R. G., Ayres, J. C., Kraft, A. A. and Forsythe, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.*, **43**: 584-595.

Breed, R. S., Murray, E. G. D. and Smith, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*, 7th ed. London, Ballière, Tindall and Cox.

Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bact.*, **52**: 461-466.

Committee on Bacteriological Technic (1957). *Manual of Microbiological Methods*. London, McGraw-Hill.

Cruickshank, R. (1965). *Medical Microbiology*, 11th ed. London, Livingstone.

Den Doreen de Jong, L. E. (1926). *Bijdrage tot de kennis van het mineralisatic proces*. Rotterdam, Nijgh and van Ditmer.

Eddy, B. P. (1960). Cephalotrichous, fermentative Gram-negative bacteria: the genus *Aeromonas*. *J. appl. Bact.*, **23**: 216-249.

Eddy, B. P. (1962). Further studies on *Aeromonas*. 1. Additional strains and supplementary tests. *J. appl. Bact.*, **25**: 216-249.

Edwards, P. R. and Ewing, W. H. (1962). *Identification of Enterobacteriaceae*, 2nd ed. Minneapolis, Burgess Publishing Co.

FLORIAN, M. L. E. AND TRUSSELL, P. C. (1957). Bacterial spoilage of shell eggs. IV. Identification of spoilage organisms. *Fd Technol. Champaign*, **11**: 56-60.

FLÜGGE, C. (1886). *Die Mikroorganismen*, 2 Aufl. Leipzig, Vogel.

FRAZIER, W. C. (1967). *Food Microbiology*, 2nd ed. London, McGraw-Hill.

HAINES, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.*, **38**: 338-355.

HAINES, R. B. (1939). Microbiology in the preservation of the hen's egg. *Spec. Rep. Fd Invest. Lond.*, no. **47**. London, H.M.S.O.

HUGH, R. AND LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.*, **66**: 24-26.

KING, E. O., WARD, M. K. AND RANEY, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. clin. Med.*, **44**: 301-307.

LAGRANGE, E. (1935). Sur la flore des oeufs pourris. *C.r. Séanc. Soc. Biol.*, **120**: 846-848.

MILES, A. A. AND HALNAN, E. T. (1937). A new species of microorganism (*Proteus melanovogenes*) causing black rot in eggs. *J. Hyg., Camb.*, **37**: 79-97.

MILES, E. M. AND MILES, A. A. (1951). The identity of *Proteus hydrophilus* Bergey *et al.* and *Proteus melanovogenes* Miles and Halnan, and their relation to the genus *Aeromonas* Kluyver and Van Niel., *J. gen. Microbiol.*, **5**: 298-306.

MILLER, W. A. AND CRAWFORD, L. B. (1953). Some factors influencing bacterial penetration of eggs. *Poult. Sci.*, **32**: 303-309.

PAVARINO, G. L. (1929). Changes produced in eggs by microorganisms. *Annali Chim. appl.*, **19**: 266-272.

PENNINGTON, M. E., JENKINS, M. K., ST. JOHN, E. Q. AND HICKS, W. B. (1914). A bacteriological and chemical study of commercial eggs in the producing areas of the middle west. *Bull. U.S. Dep. Agric.*, **51**.

PLATT, A. E. AND ANDERSON, C. F. (1939). Green rot in eggs. *J. Dep. Agric., S. Aust.*, **42**: 1040-1041.

RHODES, M. E. (1959). The characterisation of *Pseudomonas fluorescens*. *J. gen. Microbiol.*, **21**: 221-263.

RICHARD, O. AND MOHLER, A. (1950). Die Mikrobiologie der " Heu-eier ". *Mitt. Lebensn. Hyg., Bern*, **41**: 168-180.

STANIER, R. Y., PALLERONI, N. J. AND DOUDOROFF, M. (1966). The aerobic pseudomonads; a taxonomic study. *J. gen. Microbiol.*, **43**: 159-271.

THORNLEY, M. J. (1960). The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *J. appl. Bact.*, **23**: 37-52.

TISSIER, H. (1926). Microbes de la putrefaction des oeufs. *C.r. Séanc. Soc. Biol.*, **94**: 446-447.

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A Note on Actinomycetes on a Museum Specimen of Eggshell

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The mycelium and spores of actinomycetes were present in and on minute black spots that had disfigured the white eggshell of a non-parasitic cuckoo during museum storage.

A WHITE EGGSHELL from a museum store was disfigured by small black spots. The observations made during studies with electron optics are the subject of this report.

Materials and Methods

The eggshell of a non-parasitic cuckoo, collected in India at the turn of the century, was a gift from the Trustees of the British Museum.

Pieces of shell bearing small black spots on their outer surface were glued with colloidal silver (DAG 915; Achesons Colloid Co., Princes Rock, Plymouth) on to round specimen holders and sputter-coated *in vacuo* with gold. The coated specimens were examined with a Stereoscan S4 (SEM; Cambridge Scientific Instruments Ltd.) at an accelerating voltage of 10 kV.

Observations

It was evident from studies with the SEM at low magnification (×200–300) that the black spots on the outer surface of the white eggshell were roughly dome shaped. The outer surface was smooth except where it was perforated (Plate 1*a*) or ridged (Plate 1*b*) perhaps as a consequence of a dome having been squashed. Indeed the impression was gained that many of the domes had been crushed and a fracture face appeared to be a notable feature of some (Plate 1*c*). Moreover the domes rested on the outer surface of the eggshell, a narrow gap between the two being a characteristic feature (Plate 1*a*,*b*). Only a few mycelia were present on the smooth outer surface whereas many more were present within holes (Plate 1*d*) or crevices (Plate 2*a*) in the domes. At high magnification it was evident that they formed a mat in some of the crevices (Plate 2*b*). Spores were also present (Plate 1*d*). In one dome (Plate 2*c*) they resembled those (arthrospores) produced by *Streptomyces* and related organisms. In another case the spore vesicles were of the type formed by members of the Actinoplanes (Plate 2*d*).

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Discussion

There is a need to conserve collections of eggshells because public opinion or diminished populations of particular species of birds is an impediment to their replenishment. Not only are collections needed for examination of general features (shape, colour, etc.) but also to provide material for the study of fine structure. Undamaged shells are required for these purposes. It is becoming recognized that changes, particularly in the appearance of the shell's surface, may occur during long-term storage. As yet, however, there is a very limited literature on the subject of change and this discussion attempts to place our observations in a rather general context so that others may be aided in future studies.

When considering disfiguration of eggshells, there is perhaps a temptation to liken it to the 'foxing' of archival documents and prints. In a recent discussion of this phenomenon, Meynell & Newsam (1978) stressed that cultural techniques are inappropriate in studies of the causes of 'foxing' because of the likelihood of the affected material harbouring fungal spores that would have remained quiescent under all but exceptional conditions of storage. They met with success by direct observation using the light microscope with methods adopted from dermatology, or electron optics; either method showed fungal hyphae in an affected area. We were unable to demonstrate by electron optics mould hyphae on a museum specimen of a dove's eggshell stained yellowish brown, and concluded that some abiotic factor was responsible. Indeed such a conclusion is in keeping with the fact that pigeons' eggshells are largely calcite and the outer surface is not covered with organic material (Board 1974; Board *et al.* 1977) on which organisms could grow. In this example, the discoloration may have resulted from the deposition of airborne volatile materials or the migration of some substance (Fe^{3+}?) from the underlying shell membranes and any yolk/albumen which had remained after 'blowing'.

It is well known that eggshells of domestic hens are colonized by micro-organisms when stored under humid conditions. Pseudomonads can digest the glycoprotein cuticle that clothes the shell's outer surface (Board *et al.* 1979) but only if the atmosphere is maintained at an r.h. of 98% or more. Moulds such as *Aspergillus* spp. will grow at r.h.'s of *ca.* 85% and give pieces of shell a whiskery appearance within 35 d at room temperature; it has not been determined whether or not they digest the cuticle.

With the egg examined in the present study, the confinement of the actinomycetes to the black spots raises important questions about the genesis of the infection. Were we examining the remnants of an infection that obtained for a short period following the collection of the egg or an infection that had remained confined simply because some environmental factor (r.h.?) achieved a growth-promoting level for short periods at infrequent intervals? The latter possibility was considered by Meynell & Newsam (1978) also in their discussion of 'foxing'. Yet another possibility is that the surface of the shell was soiled with yolk and albumen when the egg was 'blown' and the black spots are such material colonized with actinomycetes. The latter suggestion prompts the recommendation that thorough washing of a shell be done before it is put into store and that the work is done by someone wearing gloves. It has been shown (Board *et al.* 1977) that the cuticle of the eggshell of the Guinea Fowl (*Numidia meleagris*) absorbs grease from the fingers. Not only might such grease provide substrates for micro-organisms but products of its oxidation might accentuate change in the colour of the shell.

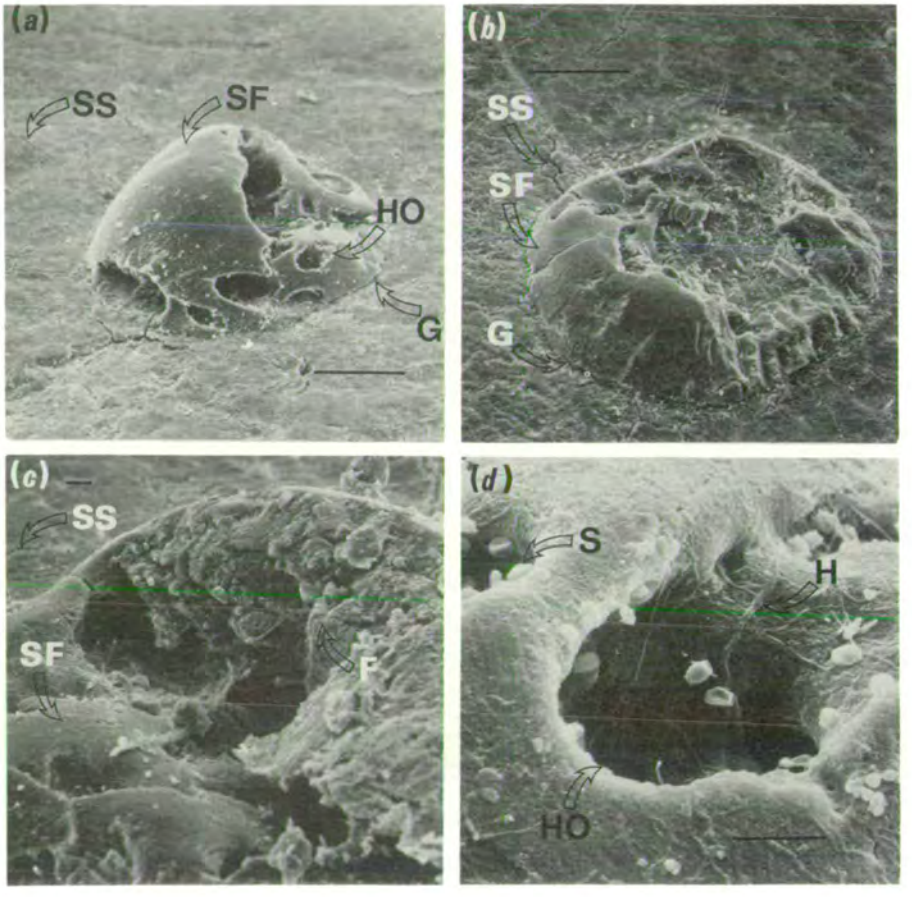

PLATE 1. General views of the black domes on the outer surface of the eggshell of a non-parasitic cuckoo [(a) marker bar, 100 μm; (b) marker bar, 100 μm, and (c) marker bar, 10 μm] and a hole [(d) marker bar, 10 μm]. SS, shell surface; G, narrow gap between periphery of dome and shell surface; SF, smooth face of dome; F, fracture face in a dome [see (c)]; HO, holes—details given in (d); H, mycelia, and S, spores (for additional details, see Plate 2*d*).

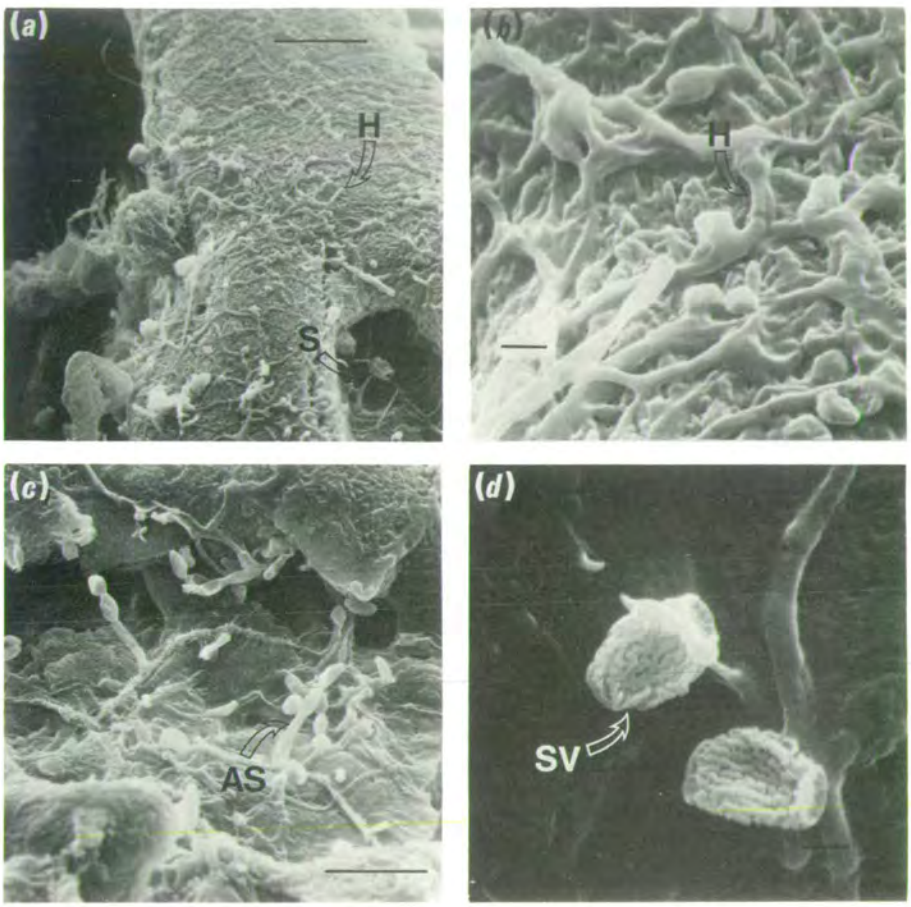

PLATE 2. (a) Details of crevice in dome showing a mat of mycelia (H) and spores (S: bar marker, 10 μm); (b) mycelia (H) on the exposed surface of a crevice in a dome (bar marker, 1 μm); (c) details of arthrospores (AS: bar marker, 10 μm), and (d) spore vesicles (SV: bar marker, 1 μm) located in the hole (HO) depicted by Plate 1*a* and *c*.

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References

BOARD, R. G. 1974 Microstructure, water resistance and water repellency of the pigeon eggshell. *British Poultry Science* **15,** 415–419.

BOARD, R. G., TULLETT, S. G. & PERROTT, H. R. 1977 A classification of the pore system in avian eggshells. *Journal of Zoology, London* **182,** 251–265.

BOARD, R. G., LOSEBY, S. & MILES, V. R. 1979 A note on microbial growth on hen eggshells. *British Poultry Science* **20,** 413–420.

MEYNELL, G. G. & NEWSAM, R. J. 1978 Foxing, a fungal infection of paper. *Nature, London* **274,** 466–468.

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A NOTE ON MICROBIAL GROWTH ON HEN EGG-SHELLS

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1. Two strains of cuticle-digesting pseudomonads were isolated from the surface of hens' egg-shells that had been stored in a humid atmosphere at 25 °C. Digestion was due to a protease, the demonstration of which was only achieved in media containing cuticle.

2. The egg-shells were colonised by yeasts also, but the growth of these organisms appeared to be dependent upon the pseudomonads for the release of nutrients from the cuticle.

3. The pseudomonads would not grow on cuticle *in situ* unless the relative humidity was about 100%.

INTRODUCTION

The accounts of microbial growth on the shells of domestic hens' eggs stored under unsuitable conditions leave no doubt about the role of a high relative humidity in promoting the growth of organisms (Weston and Halnan, 1927; Sharp and Stewart, 1936; Knowles and Clerkin, 1939; Lorah *et al.*, 1954). Indeed water would appear to be the only limiting " nutrient " when it is recalled that the shells of the majority of eggs of domestic hens (Board and Halls, 1973; Ball *et al.*, 1975) are clothed with cuticle, a stratum of spherules (Simons and Wiertz, 1963; Simons, 1971; Tullett *et al.*, 1975) composed of lipids, carbohydrates, proteins and trace metals (Wedral *et al.*, 1974). Porphyrins are present also (Baird *et al.*, 1975). The studies noted above were concerned solely with moulds because, presumably, their mycelia gave a characteristic " whiskery " appearance to the eggs. Although many of the fungi which colonise eggs have been identified (Lorah *et al.*, 1954), little attention has been given to their physiological properties, particularly the ability to digest cuticle. This communication presents evidence from a preliminary study of the role of cuticle-digesting bacteria as pioneers in a succession leading to yeasts becoming dominant.

MATERIALS AND METHODS

The eggs were obtained from commercial laying stock kept in batteries and fed on a proprietary layer diet.

Isolation of micro-organisms

Twelve hens' eggs were incubated (4 weeks at 25 °C) in a sealed container in which a saturated atmosphere was ensured by a cellulose wad saturated with water. The surface of the shell was scraped with an inoculating loop and the material streaked on plate count agar (Oxoid). Pieces of colonised shell were fixed in a solution containing 30 g gluteraldehyde/l 0·3 M phosphate buffer (pH 7·0) for 24 h. After two washings in phosphate buffer (pH 7·0), the shells were immersed for 15 min in acetone/water (500 g/l) and then three times for 30 min in pure acetone. The specimens were critical-point dried, coated *in vacuo* with silver and examined with a scanning electron microscope (Stereoscan S4, Cambridge Scientific Instruments Ltd) with an accelerating voltage of 10 kV.

Growth on, and digestion of, the cuticle

The cuticle was scraped from the surface of nest-clean, hens' eggs which had been immersed for 60 min in a solution of 50 g ethylenediaminetetracetic acid (EDTA)/l (pH 7·5). It was suspended in distilled water, ground with a hand-operated tissue homogeniser and freed of EDTA by four washings in distilled water, the fragments of homogenised cuticle being harvested by centrifugation between each washing. The cuticle was added to agar (1 g/l; Oxoid) containing the mineral salts solution (10 ml/l) of Keddie *et al.*, (1966) from which the nitrogen source was omitted or into the salts solution only. The media were sterilised at 121 °C for 15 min. The agar medium was poured as a shallow layer on an agar base containing only mineral salts solution in Petri dishes; the medium was streak-inoculated to give isolated colonies, a loopful of inoculum being taken from a nutrient broth (Oxoid) culture after 24-h incubation at 30 °C. The cuticle suspension was dispersed (100 ml) in Erlenmeyer flasks (250 ml) and inoculated with a loopful of nutrient broth (Oxoid) culture after incubation for 24 h at 30 °C. Microbial growth in the cuticle suspension was assessed by the viable counting technique of Miles and Misra (1938). Samples of the cuticle suspensions were filtered through a membrane filter with pore size 0·45 μm and the filtrate used to determine: total carbohydrate (Morris, 1948); total protein (Lowry *et al.*, 1951); proteolytic activity (Rinderknecht *et al.*, 1968), using hide powder (Hopkin and Williams) complexed with remazolbrilliant blue (Hoechst, Manchester) as substrate; and protoporphyrin concentration (Schwartze and Wikoff, 1952). The identity of cuticular protoporphyrin was confirmed by thin layer chromatography and spectral analysis (Kennedy and Vevers, 1973) using protoporphyrin IX (Sigma) as standard.

Relative humidity and microbial growth

The method of Ayerst (1969) was used. Test tubes (23 × 150 mm) were closed with a rubber bung into the small end of which was inserted a glass rod (5 × 60 mm). A piece of rubber pressure tubing, the free end of which was slit with a sharp scalpel was fixed to the glass rod. The boiling tubes and rubber bungs were autoclaved at 121 °C for 15 min separately, and assembled aseptically. Sodium chloride solutions, autoclaved similarly were prepared according to the data of Robinson and Stokes (1958) and 4 ml of an appropriate concentration added aseptically to a test tube

(capacity about 60 ml). Pieces of egg-shells (15 × 5 mm) were sterilised with ethanol water (700 g/l) and one end of a piece inserted in the slit in the free end of the pressure tubing. The tubes, together with the egg-shells, were incubated for 4 d before inoculation, the tubes being held in individual compartments of a cardboard box to insulate them from slight fluctuations in temperature. A 1 : 10 dilution of bacteria (24-h culture of the test organism incubated at 30 °C in nutrient broth (Oxoid): quarter-strength Ringer's solution) was used to inoculate the outer surface of a piece of shell. The Miles and Misra (1938) method was used to determine the viable count in a suspension prepared by grinding a piece of shell in quarter-strength Ringer's solution, plate count agar (Oxoid) being used to grow the organisms.

Staining of the cuticle

An aqueous solution (10 g/l) of Edicol supra pea green H (ICI, Hexagon House, Blackley, Manchester) was used. This stains cuticular material present on the shell (Board and Halls, 1973).

RESULTS

After 4 weeks storage at 25 °C in a humid atmosphere, the surfaces of the egg-shells were heavily blotched with white dusty material which on examination with a light microscope was found to be composed of yeasts. It was evident in SEM studies of pieces of shell prepared by the critical-point drying method that the yeasts overlaid a layer of bacterial cells, this layer extending out from the edge of the yeast colony. Staining shells with Edicol pea green and rinsing with tap water gave a preparation in which large unstained areas occurred on the egg-shell surface. This was taken as evidence that colonised cuticle had been broken down or freed from the surface of the shell as a consequence of microbial growth.

In addition to the yeast colonies, three bacterial colony forms were dominant on plate count agar which had been inoculated with material taken from colonised egg-shells. Both the yeasts and the bateria were isolated from all the eggs examined from the one batch stored for 4 weeks at 25 °C. None of the yeast isolates formed other than a faint colony on agar containing mineral salts (Keddie *et al.*, 1966) and fragments of cuticle. Indeed, the extent of their growth was comparable with that of the dominant strains of bacteria inoculated on agar containing salts solution only. Two of the bacterial isolates grew extensively on agar containing cuticle. Their growth was associated with the loss of brown colour and disintegration of the fragments of cuticle situated beneath or immediately alongside a colony. Prolonged incubation led to the breakdown of cuticle situated down in the agar. The breakdown of the cuticle was clearly evident during examination with the light microscope; early in incubation it was manifested by a " fraying " of the edges of fragments of cuticle and loss of the brown pigment; with prolonged incubation the fragments of cuticle appeared to consist of spherules. Both the isolates which brought about these changes were characterised in sufficient detail (Table 1) to identify them with *Pseudomonas*. Although their colony forms (one was lightly tinted yellow) allowed recognition, detailed characterisation did not yield results which warranted formal definition of differences.

TABLE

Properties of pseudomonads isolated from cuticle of hen eggs

Gram negative rods; motile; single polar flagellum.
Obligate aerobe; oxidase and catalase positive.
Growth at 41 and 37 but not 4 °C.
No action on glucose, fructose, maltose, sucrose, lactose.
No pigments produced.
No anaerobic growth with NO_3 as electron acceptor.

The growth of pseudomonads in a mineral salts solution containing fragments of cuticle was much more extensive than that in mineral salts solution alone (Fig. 1). Growth was associated with a break-up of the cuticle fragments and an increase in the brown colour of the medium. In one experiment there was a three-fold increase in the protoporphyrin concentration of the filtrate of the medium during 198-h incubation. In the first 48-h incubation of cuticle with pseudomonads, there was a marked increase (Fig. 1) in the protein concentration of medium which had been filtered. Subsequently there was a peak in concentration of carbohydrate in filtered medium. The peak concentration of protease in the medium occurred subsequent to the populations attaining their largest size and subsequent also to the peak in protein concentration of the filtered medium. There was a gradual increase in the carbohydrate concentration in filtered medium in the early part of incubation and the greatest concentration occurred at about the same time as the protease concentration reached its maximum. In all experiments it was notable that one of the pseudomonad isolates was more active than the other (Fig. 1). It was noted above that prolonged incubation was required before cuticle contained in agar showed signs of digestion when the bacterial colonies were not close. Additional evidence that digestion of the cuticle is confined at the outset was obtained from studies of microbial growth on the cuticle of pieces of egg-shell. It was evident from scanning electron microscope studies of such preparations that cuticle digestion was confined to the immediate vicinity of the cells (Plate-Fig. 1).

Proteolytic activity was not demonstrated in filtrates when either of the pseudomonads was grown in salts solution (complete with nitrogen source) containing lactate. The protease was demonstrable in filtrates shortly after the addition of cuticle to the medium. It has yet to be determined whether the proteases are latent in the cell and only released when the substrate is present or whether their synthesis is induced only by a substrate.

Previous investigations have linked fungal colonisation of egg-shells with storage in humid atmospheres. When eggs are stored in a sealed container, the relative humidity of the atmosphere will equilibrate with the water activity, about 0·98, of the albumen. Thus pieces of shell freed of albumen need to be used to study the influence of relative humidity (RH) on microbial growth. In the present study, the method of Ayerst (1969) was used to achieve this end. Fig. 2 shows that the growth of pseudomonads on the cuticle of egg-shells occurred in the 24 h after inoculation in test tubes, the atmospheres of which had RH's of 95 and about 100%; there was less growth on shells from which the cuticle had been removed. After 14-d incubation, the populations on shells, with or without cuticle, stored at an RH of about 100%, were the only ones which were larger than the initial

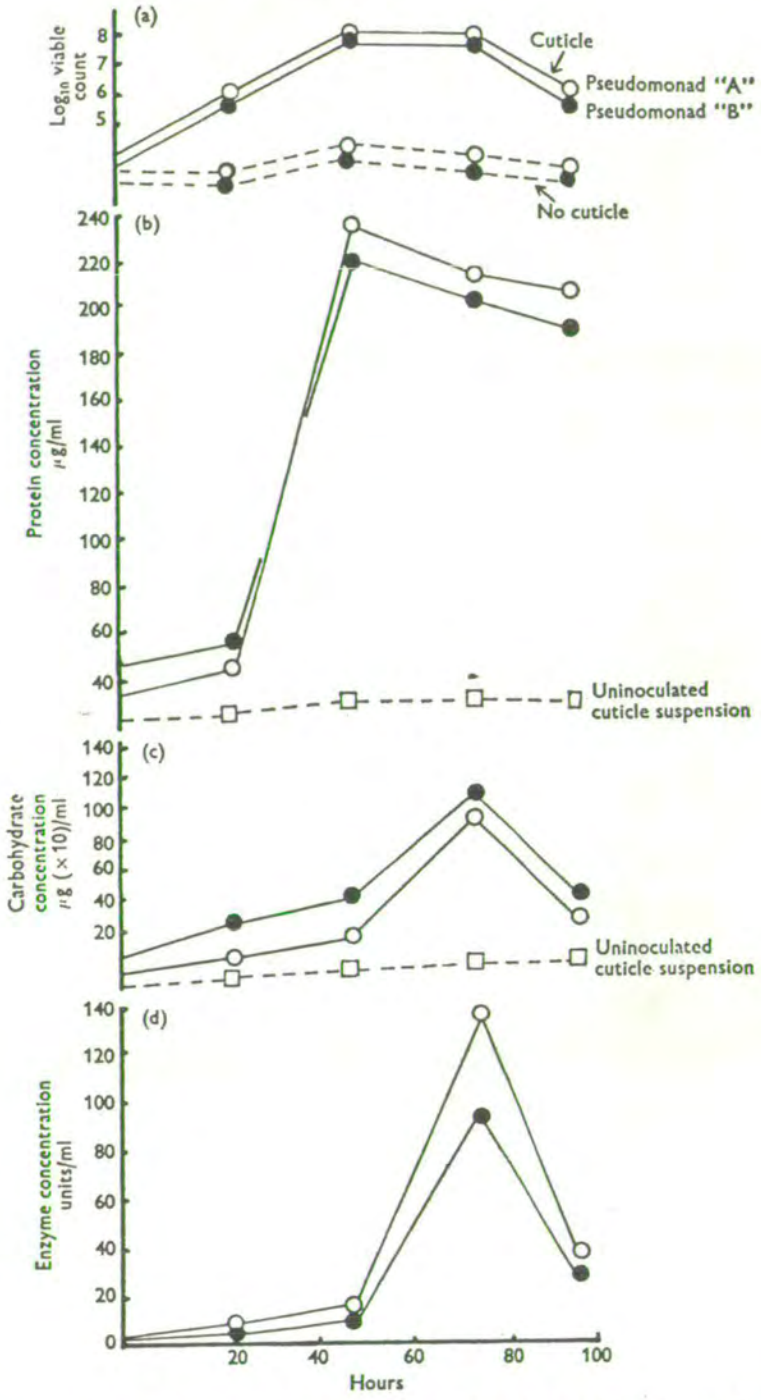

FIG. 1.—The growth on, and digestion of, cuticle by two strains (● ○) of pseudomonads. (a) Growth of pseudomonad on cuticle suspended in mineral salts solution incubated at 30 °C; (b) the protein content of filtrates (membrane filter, 0·45 μm pore size) of cuticle suspension; (c) the carbohydrate content of the filtrate, and (d) the proteolytic activity of the filtrate.

inoculum. As the growth in the 24 h after the introduction of inoculated shell into an atmosphere having a nominal RH of 95% may have occurred during the period required for equilibrium to be established between the moist shell and the atmosphere, the results in Fig. 2 suggest that an RH of about 100% is required by the pseudomonads.

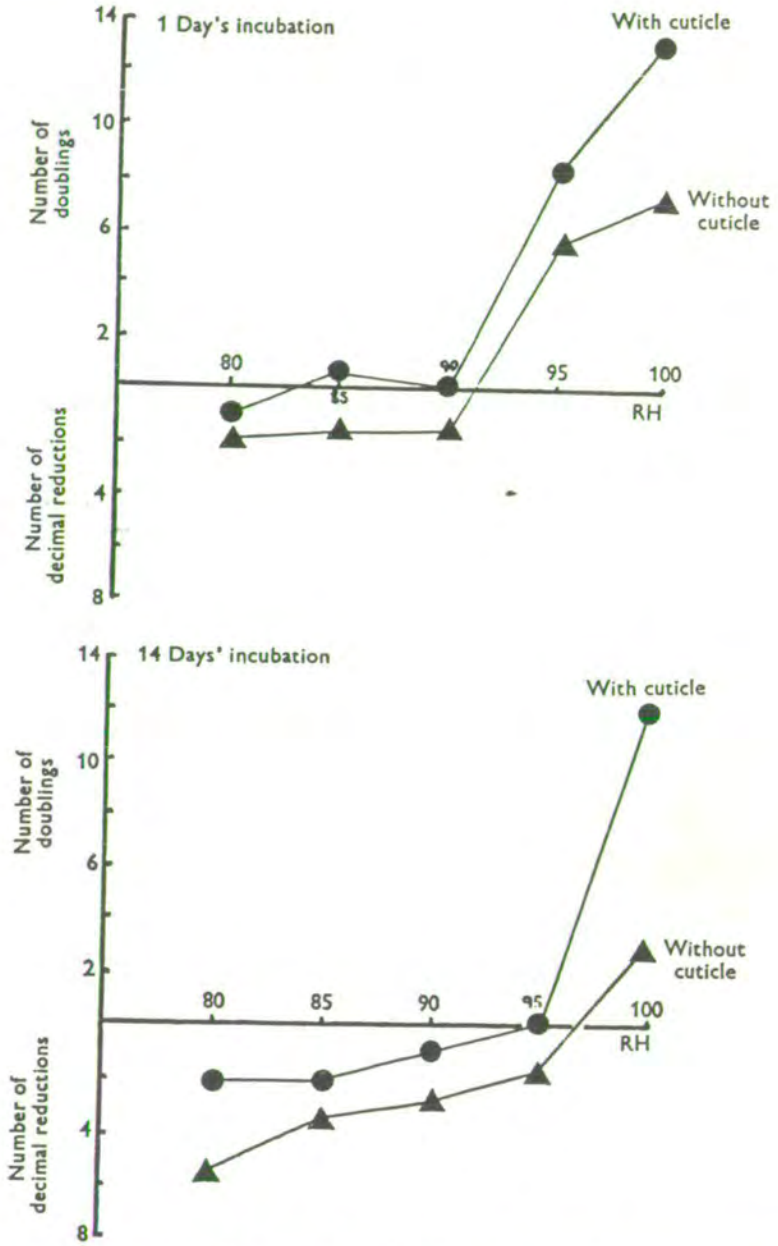

FIG. 2.—Changes in the populations of a strain of pseudomonad on egg-shells incubated at 25 °C, with or without cuticle, at different relative humidities (RH).

DISCUSSION

This study has demonstrated that the cuticle on the hens' egg can be digested when colonised during storage in a humid atmosphere. Moreover, it was noted that the yeasts which produced macroscopic changes on the surfaces of the stored eggs failed to grow on, or digest, cuticle suspended in a mineral salts solution. It would seem therefore that the growth of the yeasts on the egg-shell was dependent on the activities of the cuticle-digesting bacteria. This surmise is supported by the observation that the yeast colonies grew on top of a film of bacteria on the cuticle on the egg-shell. Final proof of the dependence of the yeasts on the activity of cuticle-digesting bacteria will have to await studies in which the growth of the former is considered alongside that of the latter. This communication presents the results of a preliminary study only and more extensive investigations might be expected to extend the list of micro-organisms, both bacteria and fungi, which have the ability to digest cuticle. The major point arising from the present investigation is the need in future studies to establish whether or not an organism isolated from a colonised egg-shell has the capacity to digest the cuticle so that its role as a pioneer or a member of a succession can be identified.

Although we have demonstrated that bacteria can digest the cuticle, the observations on the isolates' response to RH, and by implication with the water activity of the cuticle, emphasise yet again the important role which the lack of suitable levels of moisture plays in protecting the hens' egg from infection of the shell. Thus, by implication, the humidity of the atmosphere around an egg should be considered as one of the components (Board and Fuller, 1974) that contribute to the antimicrobial defence of the developing avian embryo.

REFERENCES

Ayerst, A. (1969). The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research*, **5**: 127-141.

Baird, T., Solomon, S. E. and Tedstone, D. R. (1975). Localisation and characterisation of eggshell porphyrins in several avian species. *British Poultry Science*, **16**: 201-208.

Ball, R. F., Logan, V. and Hill, J. F. (1975). Factors affecting the cuticle of the egg as measured by intensity of staining. *Poultry Science*, **54**: 1479-1484.

Board, R. G. and Fuller, R. (1974). Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biological Reviews*, **49**: 15-49.

Board, R. G. and Halls, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the hen's egg. *British Poultry Science*, **14**: 69-97.

Keddie, R. M., Leask, B. G. S. and Grainger, J. M. (1966). A comparison of coryneform bacteria from soil and herbage: cell wall composition. *Journal of Applied Bacteriology*, **29**: 17-43.

Kennedy, G. Y. and Vevers, H. G. (1973). Egg-shell pigments of the Araucano fowl. *Comparative Biochemistry and Physiology*, **44B**: 11-25.

Knowles, N. R. and Clerkin, P. (1939). Investigations of an instance of mould infection in sealed eggs. *Journal of the Ministry of Agriculture, Northern Ireland*, **6**: 63-68.

Lorah, M. E., Funk, E. M. and Forward, J. (1954). Reducing spoilage in shell eggs by the use of fungicides. *College of Agricultural Experimental Station, University of Missouri Research Bulletin*, **573**.

Lowry, O. H., Rosebrough, N. F., Farr, A. L. and Randall, R. F. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**: 265-268.

Miles, A. A. and Misra, S. S. (1938). The estimation of the bactericidal power of blood. *Journal of Hygiene, Cambridge*, **38**: 732-749.

MORRIS, D. L. (1948). Colorimetric determinations of carbohydrate with Dreywood's anthrone reagent. *Science, New York*, **107**: 254-255.

RINDERKNECHT, H., GEOKAS, M. C., SILVERMAN, P. AND HAVERBACK, B. F. (1968). A new ultrasensitive method for the determination of proteolytic activity. *Clinica Chimica Acta*, **21**: 197-203.

ROBINSON, R. A. AND STOKES, R. H. (1959). *Electrolyte Solutions*, 2nd ed. London, Butterworth.

SCHWARTZE, S. AND WIKOFF, H. M. (1952). The relation of erythrocyte coproporphyrin and protoporphyrin to erythropoiesis. *Journal of Biological Chemistry*, **194**: 563-573.

SHARP, P. F. AND STEWART, G. F. (1936). Effect of relative humidity on the growth of mold on eggs in storage. *Cornell Agricultural College, Experimental Memoirs*, **191**: 1-110.

SIMONS, P. C. M. (1971). Ultrastructure of the hen eggshell and its physiological interpretation. *Centre for Agriculture Publishing and Documentation, Wageningen.*

SIMONS, P. C. M. AND WIERTZ, G. (1963). Notes on the structure of membranes and shell in the hen's egg. An electron microscopical study. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, **59**: 555-567.

TULLETT, S. G., LUTZ, P. L. AND BOARD, R. G. (1975). The fine structure of the pores in the shell of the hen's egg. *British Poultry Science*, **16**: 93-95.

WEDRAL, E. M., VADEHRA, D. V. AND BAKER, R. C. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of *Gallus gallus*. *Comparative Biochemistry and Physiology*, **47B**: 631-640.

WESTON, W. A. R. D. AND HALNAN, E. T. (1972). Black spots of eggs. *Poultry Science*, **6**: 251-258.

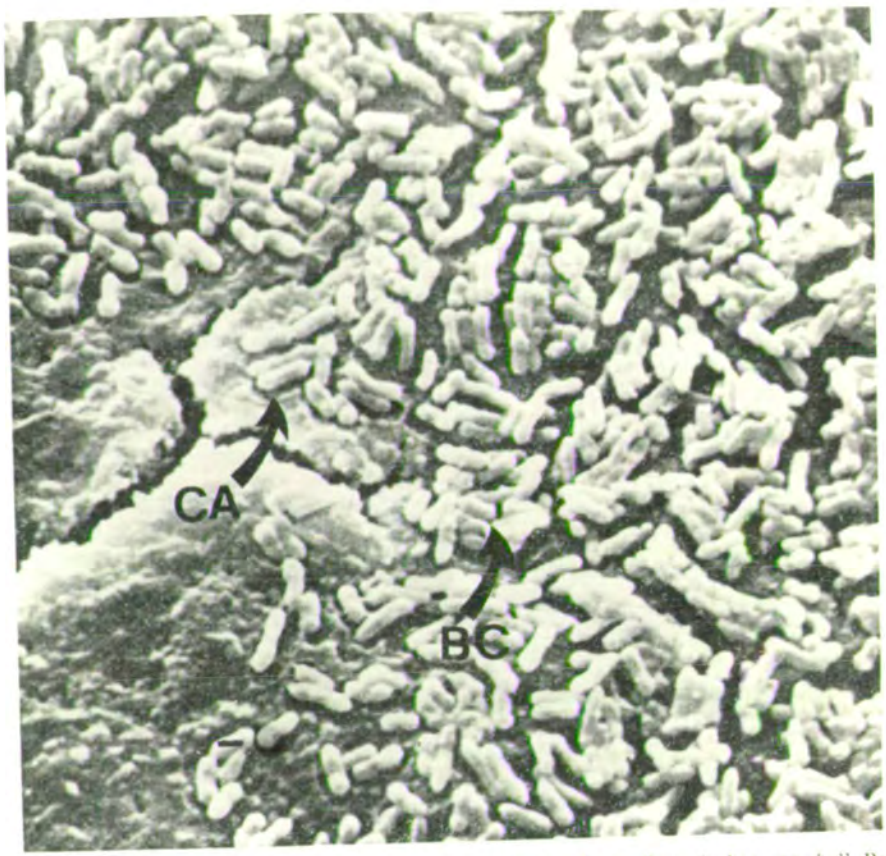

PLATE-FIG. 1.—Digestion of cuticle by a pseudomonad growing on the surface of a hen egg-shell. Bacterial cells (BC) surrounded by a cleared area (CA) of cuticle. The inoculated shell had been incubated at 30 °C for 5 d. × 12 000.

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Bacterial Growth on and Penetration of the Shell Membranes of the Hen's Egg

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Summary. Commonly occurring contaminants of rotten eggs multiplied in a buffered solution of mineral salts containing intact shell membranes. *Aeromonas liquefaciens*, a nonpigmented pseudomonad, and sometimes a proteolytic strain of a *Cloaca* sp. caused the membranes to lose their pink colour. This was associated with a marked increase in the turbidity of the suspending medium and with the appearance of substances which reacted with Nessler's reagent and ninhydrin. These changes were not seen after growth of *Proteus vulgaris*, *Alcaligenes faecalis*, *Pseudomonas fluorescens* or non-proteolytic strains of *Cloaca* spp. No differences were noted in the rate of penetration of the shell and shell membranes by these two groups.

Shell membranes *in situ* of hen's eggs can act as a bacterial filter (Haines & Moran, 1940) but this property is lost during contact with bacteria for 18–24 h (Walden, Allen & Trussell, 1956; Garibaldi & Stokes, 1958). The reason for this is not understood. No adequate explanation has emerged from several investigations of bacterial growth on shell membranes *in vitro* (Stuart & McNally, 1943; Stokes & Osborne, 1956; Elliot & Brant, 1957; Garibaldi & Stokes, 1958), but a possible reason is that in all of these studies comminuted membranes were used. The major component of the membrane is keratin, and it has been shown by Noval & Nickerson (1959) that keratin structures may be attacked by certain organisms only if the structures are first damaged mechanically.

The present investigation was undertaken with the object of obtaining information on the extent of growth of the commonly occurring contaminants of rotten eggs in a solution of mineral salts containing shell membranes which had suffered minimum damage. In addition evidence was sought of bacterial digestion and penetration of the membranes.

Materials and Methods

Organisms

All the organisms were isolated by the author from eggs which had rotted on the premises of the producer or under controlled conditions in the laboratory. They were characterized in detail and typical representatives of the numerically predominant contaminants of rotten eggs were used in this study. This collection contained the following: *Alcaligenes faecalis*, *Pseudomonas fluorescens*, *Aeromonas liquefaciens*, *Proteus vulgaris*, *Cloaca* spp. and a nonpigmented pseudomonad. In all experiments, an inoculum was obtained from 9 ml of distilled water to which had been added 1 drop from a capillary pipette of an 18 h nutrient broth culture incubated

at 27°. The nutrient broth was composed of (% w/v): peptone (Evans), 0·25; peptone (Difco), 0·25; Lab-Lemco (Oxoid), 0·5; NaCl, 0·25; pH 7; in tap water.

Growth on shell membranes

Naturally clean eggs obtained from a mated flock of White Leghorns were used within 4 days of laying. The eggs were cracked across their equator, the contents discarded and the inside of the shell flushed with tap water. Great care was taken to minimize tearing the membranes during their removal from the shell. The membranes were again washed in water and then sterilized by holding in a jar containing about 5 ml of chloroform for 24 h, after which the chloroform was removed in the air stream generated by a water pump. The membranes from 1 egg, or pieces from 6 which approximated the amount present in 1 egg, were introduced aseptically into a 50 ml Erlenmeyer flask containing 15 ml of a medium similar to that of Garibaldi & Stokes (1958). It consisted of a M/15 Sørensen's buffer containing the following (mg/l): $MgSO_4.7H_2O$, 200; $ZnSO_4$, 5; $MnCl_2.4H_2O$, 7·2; $FeSO_4.7H_2O$, 10; $CaCl_2$, 11; $CoCl_2$, 1·0. The pH was checked after the medium had been sterilized by holding momentarily at 128° (22·5 lb steam pressure/in^2). The membrane suspension was inoculated with either 1 or 5 drops of the dilute suspension of organisms. In practice, this meant that the initial size of the populations was either *c.* 1000 or *c.* 10,000 organisms/ml of suspending medium. These two levels were used with all of the organisms included in this study. The method of Miles & Misra (1938) was used to follow changes in the numbers of viable organisms during incubation at 27°, the procedure being to prepare a series of decimal dilutions in 9 ml of quarter-strength Ringer's solution and inoculate 0·02 ml of each on the surface of a nutrient agar (nutrient broth containing 1·5% (w/v) agar). The plates were incubated at 27° until the size of the colonies was optimal for enumeration.

Digestion of shell membranes

Both Nessler's reagent and the photometric ninhydrin method of Moore & Stein (1948) were used to measure the products of protein digestion in the suspending medium. Ninhydrin (British Drug Houses; Laboratory Reagent) was purified by treatment with charcoal and recrystallized. The intensity of the ninhydrin colour was measured in a Spekker absorptiometer with the yellow filter 606.

Penetration of the shell membranes

This was examined by a method similar to that of Garibaldi & Stokes (1958) in the apparatus shown in Fig. 1. The shells were wiped with 70% ethanol, the pointed ends removed and the contents discarded. Each shell, held in a pair of sterile forceps, was again wiped with ethanol before being placed in a crystallizing dish containing 15 ml of an 18 h nutrient broth culture of the test organism. Fifteen ml of sterile nutrient broth was introduced to the inside of the shell. The apparatus was incubated at 27° and penetration of the shell and purity of the culture was tested at 24 h intervals by streaking a loopful of the broth from inside the shell on nutrient agar. Each organism was tested with a minimum of 3 eggs.

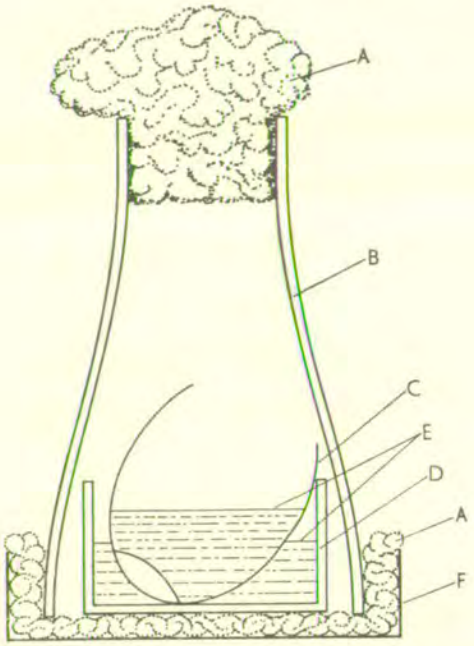

Fig. 1. Apparatus used in the investigation of bacterial penetration of the egg shell and shell membranes.
A, cotton wool; B, top of a milk bottle; C, egg shell; D, crystallizing dish; E, nutrient broth; F, top of a Petri dish.

Results

Growth on the shell membranes

Some of the results with different organisms are illustrated in Fig. 2. Multiplication occurred in the suspending medium without membranes in the majority of instances but it was always less than that occurring in the suspension of membranes. It is noteworthy that *A. faecalis*, which is nonproteolytic, multiplied to the same extent as the proteolytic organisms. The final population appeared to be determined by the size of the inoculum and it was thought that this may have been due to nutrients derived from the mother culture. This possibility was tested by comparing the growth of a nonpigmented pseudomonad in a suspension of membranes with growth in the same medium supplemented with trace amounts of peptone, yeast extract, deep litter material, etc. The supplements clearly increased the rate of multiplication in the period immediately following inoculation, but did not have a marked effect on the final size of the population (Fig. 3). These experiments indicate that undamaged shell membranes contain substances able to support the growth of the commonly occurring contaminants of rotten eggs. The results obtained with suspensions containing small amounts of peptone, yeast extract, etc. suggest that the growth substances contained in the shell membranes are not readily available to the micro-organisms.

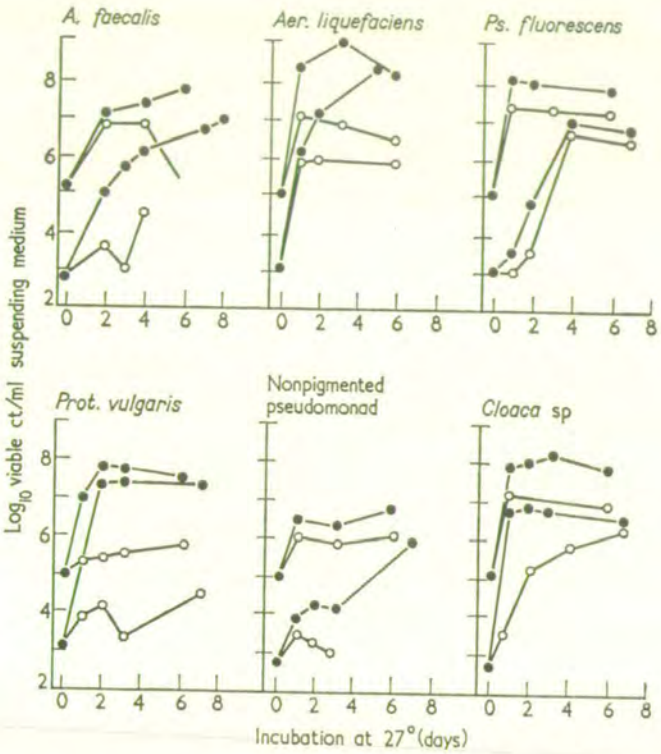

Fig. 2. Growth of bacteria at two levels of inoculum in a mineral salts medium containing the shell membranes of one egg.

Closed circles, suspension of membranes; open circles, suspending medium without membranes.

Digestion of the shell membranes

Changes in the gross structure of the shell membranes were not noted in the experiments discussed above but there was a disappearance of the pink tinge in membranes inoculated with certain organisms. The pigment, the chemistry of which is in doubt (Brooks, pers. comm.), is seen in shell membranes after they have been exposed to the atmosphere and it was retained by uninoculated membranes throughout incubation. No loss of colour occurred in membranes inoculated with *A. faecalis*, *Ps. fluorescens* or *Prot. vulgaris*. The pigment was lost occasionally from membranes inoculated with a proteolytic strain of *Cloaca* and invariably when *Aer. liquefaciens* or a nonpigmented pseudomonad were used. This loss did not appear to be due to a change in pH since the value attained in an uninoculated suspension of membranes (pH 7·6) was not significantly different from those of inoculated suspensions (pH 7·6–8·0). The turbidity of cultures containing decolorized membranes was much heavier than that in the medium without membranes or in cultures containing non-decolorized membranes. The results obtained when Nessler's reagent was added to the suspending medium are summarized in Table 1, from which it will be seen that

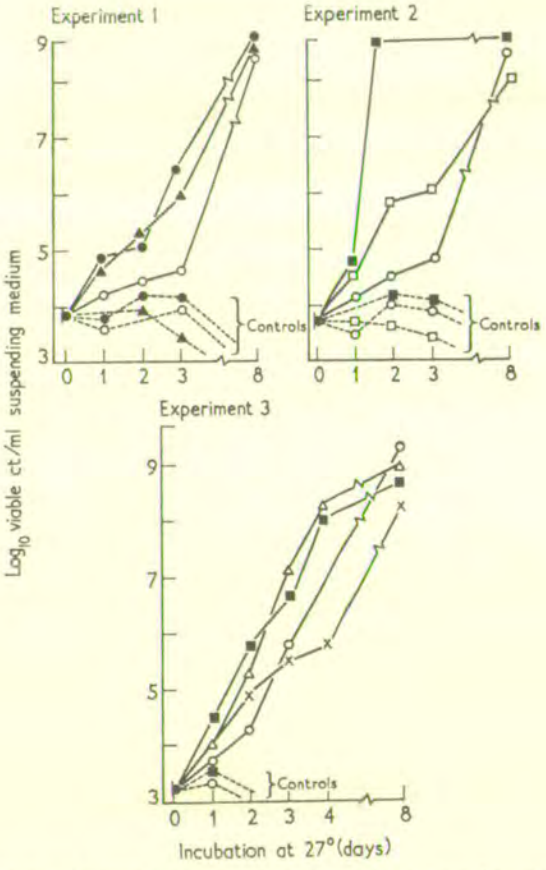

Fig. 3. The influence of supplements on the growth of a nonpigmented pseudomonad in suspensions of shell membranes.

Entire lines, suspensions of membranes with or without supplements; broken lines, control suspending medium with or without supplements. The supplements, used in a final concentration of 0·01% (w/v), were: none (open circles); hens' faeces (closed circles); deep litter (closed triangles); yeast extract (closed squares); ash of yeast extract (open squares); peptone (open triangles) and glucose (crosses).

positive results were obtained only with turbid cultures containing decolorized membranes: the atmosphere above the latter contained H_2S. The results obtained from experiments in which ninhydrin was used to measure the release of substances from the shell membranes (Fig. 4) show that ninhydrin-reacting substances were detected on the first or second day following inoculation and that a peak was reached several days later. The subsequent decrease in the concentration of these substances was presumably due to the breakdown of amino acids.

The foregoing observations led to an examination of the suspending medium for the presence of proteolytic enzymes. One ml of the suspending medium was mixed with a drop of chloroform and then used to fill holes (7 mm diam) cut in water agar (1·5 g of agar in 100 ml of water) containing 0·4% (w/v) of gelatin and a crystal of

TABLE 1

Changes occurring in suspensions of shell membranes inoculated with commonly occurring contaminants of rotten eggs*

Organism	Reactions in: Suspending medium plus shell membranes† — Turbidity‡	Proteolytic§ enzymes	Reaction with Nessler's Reagent	Appearance of membranes	Suspending medium alone — Turbidity	Proteolytic enzymes	Reaction with Nessler's Reagent
A. faecalis	+ +	Absent	–	Unchanged	+	Absent	–
Ps. fluorescens	+ +	Absent	–	Unchanged	+	Absent	–
Prot. vulgaris	+ +	Absent	–	Unchanged	+	Absent	–
Cloaca sp.	+ + +	Present	+	Decolorized	+	Absent	–
Aer. liquefaciens	+ + +	Present	+	Decolorized	+	Absent	–
Nonpigmented pseudomonad	+ + +	Present	+	Decolorized	+	Absent	–

* The recordings were made after 5 days at 27°. † The membranes from one egg were suspended in 15 ml of a buffered (pH 7·2) solution of mineral salts. ‡ Visual estimation: +, slight; + +, light; + + +, heavy. § Digestion in 24 h at 37° of gelatin around holes cut in a water agar containing gelatin, thymol and chloroform was taken as evidence of the presence of proteolytic enzymes in the suspending medium placed in the holes.

thymol. The Petri dishes were incubated for 24 h at 37° and then flooded with acidified mercuric chloride. The absence of opacity around the holes was taken as evidence of the action of proteolytic enzymes. It will be seen from Table 1 that these enzymes were present only in a medium which contained decolorized membranes. Thus shell membranes suspended in a buffered solution of mineral salts

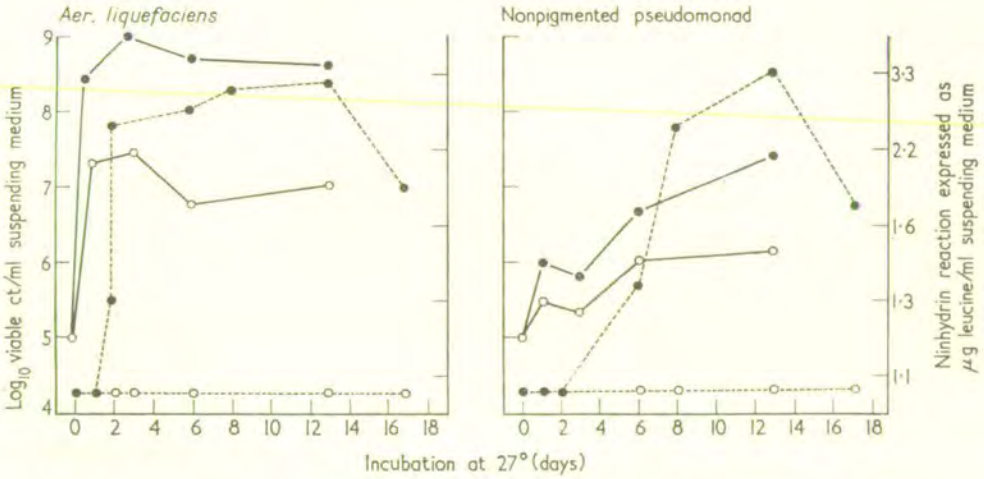

Fig. 4. The release of ninhydrin-reacting substances from shell membranes during the growth of bacteria.

Closed circles, solution of mineral salts containing shell membranes (6 pieces from separate eggs which together approximated in area the membranes from half an egg); open circles, suspending medium without membranes. Entire lines, viable counts; broken lines, ninhydrin reaction. Uninoculated suspensions of membranes did not give a measurable reaction with ninhydrin.

appear to provide an environment in which only some of the commonly occurring proteolytic contaminants of rotten eggs can synthesize proteases. The medium was evidently inadequate for *Prot. vulgaris* and proteolytic strains of *Ps. fluorescens* and it was not improved by the addition of calcium ions or pieces of shell.

Bacterial penetration of the shell membranes

In the method used to examine penetration of the membranes (Fig. 1), the external surfaces of the shell and shell membranes were exposed to large populations of the test organism throughout incubation. The results given in Table 2 show a variation in the time taken for individual shells to be penetrated by the same organism. This was common to all the organisms examined. In order to compare the rate of

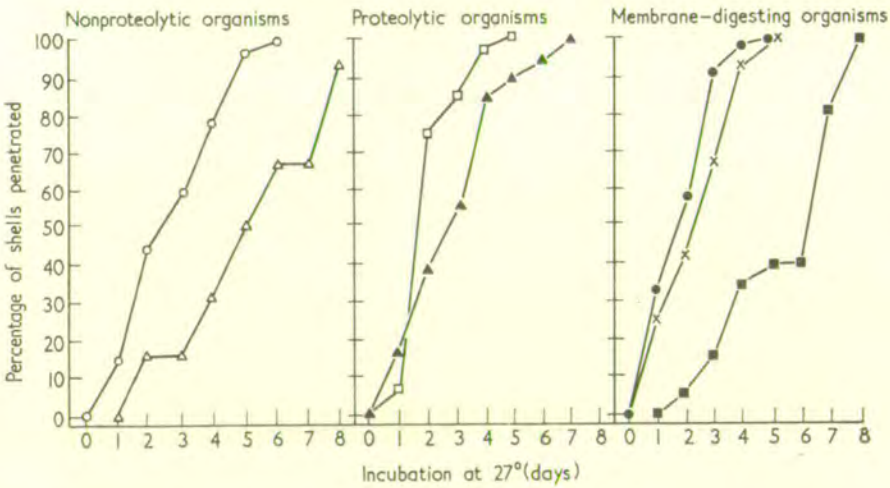

Fig. 5. Bacterial penetration of the shell and shell membranes.
The organisms tested were: nonproteolytic *Cloaca* sp. (open circles); *A. faecalis* (open triangles); *Ps. fluorescens* (open squares); *Prot. vulgaris* (closed triangles); proteolytic *Cloaca* sp. (closed circles); *Aer. liquefaciens* (crosses) and a non-pigmented pseudomonad (closed squares).

TABLE 2

Penetration of the shell and shell membranes by Prot. vulgaris *G16*

No. of eggs examined	Period of incubation at 27° (h)	Progressive no. of shells penetrated
8	24	2
	48	4
	72	4
	96	5
	120	6
	144	7
	168	8

penetration of the membranes by nonproteolytic, proteolytic and membrane-digesting organisms the results obtained with the strains of each species were combined. There was no marked difference in the rates of penetration by the three groups, although inexplicably *Ps. fluorescens* appeared to be the most active (Fig. 5). However, these organisms did not digest the membranes *in vitro* and, in view of the results obtained with *Aer. liquefaciens*, penetration of the membranes is unlikely to be assisted by polar flagellation.

Discussion

Several investigators have reported that a suspension of comminuted shell membranes can support the multiplication of the commonly occurring contaminants of rotten eggs (Stuart & McNally, 1943; Stokes & Osborne, 1956; Elliot & Brant, 1957; Garibaldi & Stokes, 1958). The present study has shown that multiplication occurs also when the membranes have not been subjected to extensive mechanical damage. It is known that organisms of the type common in rotten eggs will proliferate in phosphate buffer and distilled water. Moreover, it has been shown that the viable count is an unsatisfactory method of determining the nutritional qualities of a medium (Ecker & Lockhart, 1961). Thus an assessment of the significance of multiplication in relation to the overall process of microbial infection of the egg must be approached with caution.

Brooks (1960) has shown that the rate and extent of multiplication of a pseudomonad in albumen *in vitro* can be increased by adding pieces of shell membrane. This could be attributed to the nutrients contained in the membranes and/or the protection afforded the organisms against the antimicrobial properties of the albumen by the network structure of the membranes—a situation analogous to the "nidus effect" with anaerobes (Knight, 1941). Such an interpretation is in agreement with the results obtained from experiments with whole eggs (Brooks, 1960; Board, 1964). In the latter experiments bacterial multiplication occurred in the membranes *in situ* but the extent of multiplication was determined by the nature of the substances deposited on the membranes, and the confinement to the membranes was due to the antimicrobial action of the albumen. Thus it would seem that the substances which allow bacterial multiplication to take place in a membrane suspension will be available to organisms in the initial stages of infection of an egg but the extent of their utilization is determined by the nature of the substances which accompany the organisms onto the membranes.

The release of ninhydrin-positive substances was used as an index of bacterial digestion of the shell membranes by Garibaldi & Stokes (1958), who worked with organisms isolated from Canadian eggs by Florian & Trussell (1957), a collection which contained essentially the same range of organisms as that used in the present study. They found only one organism, *A. bookeri*, which released ninhydrin-reacting substances from the membranes. This organism has many properties in common with the nonpigmented pseudomonad which produced ninhydrin-positive substances from the undamaged membranes in the present study. *Aer. liquefaciens* and a proteolytic strain of a *Cloaca* sp. are other organisms which have now been shown to digest the membranes. Thus, although a suspension of lightly damaged shell membranes

will support the growth of the commonly occurring contaminants of rotten eggs, it does not seem to provide an environment in which all proteolytic bacteria can synthesize proteases. Moreover the results obtained from the experiments on bacterial penetration of the shell and shell membranes do not suggest that bacterial proteases play an important part in the process of invasion. The results and the conclusions of the present work are in accord with those of Garibaldi & Stokes (1958).

The author wishes to thank Dr. T. Gibson for his invaluable help and guidance and the British Egg Marketing Board for financial support.

References

Board, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.* **27**, 350.

Brooks, J. (1960). Mechanism of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.* **23**, 499.

Ecker, R. E. & Lockart, W. R. (1961). Relationship between initial nutrient concentration and total growth. *J. Bact.* **82**, 80.

Elliott, L. E. & Brant, A. W. (1957). Effect of saline and egg shell membrane on bacterial growth. *Food Res.* **22**, 241.

Florian, M. L. E. & Trussell, P. C. (1957). Bacterial spoilage of shell eggs. IV. Identification of spoilage organisms. *Food Tech., Champaign* **11**, 56.

Garibaldi, J. A. & Stokes, J. L. (1958). Protective role of shell membranes in bacterial spoilage of eggs. *Food Res.* **23**, 283.

Haines, R. B. & Moran, T. (1940). Porosity of, and bacterial invasion through, the shell of the hen's egg. *J. Hyg., Camb.* **40**, 453.

Knight, B. C. J. G. (1941). Factors in the cultivation of anaerobic bacteria. *J. Soc. chem. Ind.* **60**, 369.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of blood. *J. Hyg., Camb.* **38**, 752.

Moore, S. & Stein, W. H. (1948). Photometric ninhydrin method for use in chromatography of amino acids. *J. biol. Chem.* **176**, 367.

Noval, J. J. & Nickerson, W. J. (1959). Decomposition of native keratin by *Streptomyces fradiae*. *J. Bact.* **77**, 251.

Stokes, J. L. & Osborne, W. W. (1956). Effect of the egg shell membrane on bacteria. *Food Res.* **21**, 264.

Stuart, L. S. & McNally, E. H. (1943). Bacteriological studies on the egg shell. *U.S. Egg Poult. Mag.* **49**, 28.

Walden, C. C., Allen, I. V. F. & Trussell, P. C. (1956). The role of the egg shell and shell membranes in restraining the entry of microorganisms. *Poult. Sci.* **35**, 1190.

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The Growth of Gram-Negative Bacteria in the Hen's Egg

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SUMMARY. Bacteriological and chemical methods were used to follow the course of infection in eggs, incubated at 27°, the air cells of which had been inoculated with a suspension of washed bacteria. In the 3–4 days following inoculation, limited bacterial multiplication occurred in the inner membrane of the air cell but very few organisms entered the albumen. These populations then remained static or decreased slightly until renewed multiplication occurred 12–30 days after inoculation. This was induced by contact of the yolk and the shell membranes: it occurred on the 12–20th day in eggs in which the yolk moved towards the site of inoculation, but later when the yolk moved in the opposite direction. At this time there was a general infection of the egg contents and significant changes occurred in the pH and glucose concentration in the albumen. In eggs that had been inoculated with chromogenic and/or proteolytic bacteria, the first macroscopic changes of the contents were seen at this time. The rate and extent of the initial multiplication was influenced by the composition of the fluid used to suspend the washed bacteria and, in all instances, the fastest multiplication occurred when iron was added to the inoculum. Moreover, renewed multiplication occurred when iron was added to the albumen of eggs in which the bacteria were in the stationary phase.

ALTHOUGH MICROBIAL infection of the hen's egg has been the subject of many investigations, it is only in the last decade that an understanding of the course of bacterial infection has begun to emerge. This attended the adoption of a technique (Elliott, 1954; Brooks, 1960) which allowed the process to be followed in the intact egg.

Many investigators have noted a lag of 10–20 days between infection of the shells of newly laid eggs and the occurrence of organisms or macroscopic changes in their contents (Zagaevsky & Lutikova, 1944; Gillespie & Scott, 1950; Bigland & Papas, 1953; Stokes, Osborne & Bayne, 1956; Orel, 1959; Fromm & Monroe, 1960; Garibaldi & Bayne, 1960). Brooks (1960), who examined eggs which had been inoculated with *Pseudomonas* spp., noted that bacterial multiplication was confined to the shell membranes during this period. The rate of bacterial multiplication increased during a second phase the beginning of which was determined by the age of an egg at the time of inoculation. This second phase was associated with gross contamination of the contents and the first evidence of rotting. With newly laid eggs, the second phase began on the 12th day following inoculation but this period was markedly reduced if the eggs were held for 7–14 days before inoculation. The induction of the second phase of multiplication has been attributed (Brooks, 1960) to a spontaneous change in the properties of the shell membranes.

The work to be described was undertaken with the object of obtaining a clearer understanding of the mechanisms responsible for this induction.

Materials and Methods

Eggs

These were produced by a mated flock of White Leghorns. The hens were kept on deep litter and fed a layers' ration. Eggs produced during one day were used in any one experiment and they were stored at room temperature before inoculation. All the eggs were candled and those of poor interior quality discarded.

Organisms

The majority of the organisms used in this work had been isolated from eggs which had rotted either on the premises of the producer or under controlled conditions in the laboratory. They were characterized in detail. In addition bacteria obtained from various collections were used for comparative purposes. None of the organisms was sensitive to the lytic action of lysozyme but, in all instances, colony formation in 10 parts of water agar (2% (w/v) agar in distilled water) containing 1 part of egg albumen was expedited by the addition of iron. An 18 h nutrient broth culture was used in all experiments. The bacteria were harvested by centrifugation, washed twice in M/15 Sørensen's phosphate buffer (pH 7·2) and finally resuspended in distilled water, or, for certain experiments, in other fluids which are mentioned in the text.

Inoculation of eggs

The outline of the air cell was marked on the shell with a pencil at the time of candling. A carborundum disk was used to drill a hole in the shell (previously swabbed with 70% ethanol) above the air cell. The needle of an hypodermic syringe was pushed through the outer shell membrane and 0·1 ml of a bacterial suspension or, in the case of controls, 0·1 ml of the suspending medium was expelled from the syringe. The hole in the shell was sealed with sterile petroleum jelly and the eggs were held in moulded trays during incubation at 27°.

Sampling

The eggs were candled and particular attention paid to the freedom of movement of the yolk, and the presence of a running air cell which occurred if the hypodermic needle had punctured the inner membrane of the air cell. Eggs showing this last condition were discarded. The shells of acceptable eggs were then swabbed with 70% ethanol, their pointed ends fractured and the contents poured into a Petri dish. The whole of the albumen was transferred to a 50 ml Erlenmeyer flask containing 10 glass beads and shaken for 5 min on a flask shaker (Microid—Griffin and Tatlock, Ltd., London). Serial decimal dilutions in quarter-strength Ringer's solution were prepared from the homogenized albumen and 1 ml samples were plated in nutrient agar containing 0·05% (w/v) iron citrate. The latter was added to counteract any inhibition of bacterial growth that might be caused by the chelating agent, conalbumin, present in the albumen. The nutrient agar was composed of 1% (w/v) peptone (Evans, Liverpool); 1% (w/v) Lab-Lemco (Oxoid Ltd.); 2·0% agar (Oxoid) in distilled water.

The shell membranes were freed from albumen by repeated flushing with sterile water and the diameter of the air cell was measured with a pair of screw-adjusted callipers. The inner membrane was then excised along its boundary of contact with the outer membrane and the disk of membrane so obtained was ground to a paste with a pestle in a mortar containing sterile sand (the pestle and mortar were sterilized with formaldehyde solution and flushed with sterile water). The paste obtained was suspended in 9 ml of quarter-strength Ringer's solution and a series of decimal dilutions prepared. Standard volumes (0·02 ml) of these were inoculated on nutrient agar by the method of Miles & Misra (1938). The plates were incubated at 27° until the size of the colonies was optimal for enumeration.

Chemical methods

The pH of albumen was determined with a glass electrode. The amount of glucose in albumen was determined in the following manner. Ten ml of albumen were added slowly to 25 ml of absolute ethanol. The coagulum was disintegrated by shaking and, after 24 h at room temperature, the mixture was filtered. The filtrate was made up to 50 ml with water in a volumetric flask and two 5 ml amounts of this were used to determine free glucose by the method of Somogyi (1945). The accuracy of this method was not checked and reliance was placed on the differences occurring between the glucose concentrations in inoculated and those in uninoculated eggs.

Results

Influence of the yolk on bacterial multiplication

The organization of an egg changes during storage. Evaporation causes an enlargement of the air cell and an increase in the density of the albumen. The yolk, as a result of absorbing water, becomes more buoyant and, because of the deterioration of the thick white, its freedom of movement is increased. A useful indication of increased movement of the yolk was provided by the speed and extent of gyration of the yolk on turning the egg during candling. Both these reached their maxima on the 7–10th day of incubation at which time, also, the yolk finally came to rest in the uppermost part of the egg.

The following experiments were designed with the object of ascertaining the influence of these changes on the course of bacterial infection of an egg. The air cells of 10 dozen 3-day old eggs were inoculated with 0·1 ml of a suspension of washed bacteria. Half the eggs were incubated (27°) with their air cells uppermost; the remainder were held in the reverse position. At frequent intervals, 5 eggs were randomly selected from each group and examined by the methods described earlier.

The results (Fig. 1 and Table 1) obtained with *Pseudomonas fluorescens* CF21 exemplify the sequence of events following inoculation with each of the organisms examined. It will be seen from Fig. 1 that in both groups of eggs there was a gradual increase in the size of the populations in the inner membrane of the air cell in the 4 days following inoculation. During this period a limited invasion of the albumen was detected in 2 eggs only (Table 1). Thereafter the sequence of events in the two

groups of eggs differed. With eggs having the air cell uppermost there was no appreciable change in the population in the inner membrane of the air cell until the 9–14th day (i.e. the 12–17th following laying). At this time the eggs could be placed in one of two groups: first, those in which there was no significant change in the number of organisms in the membrane of the air cell and in which there was no

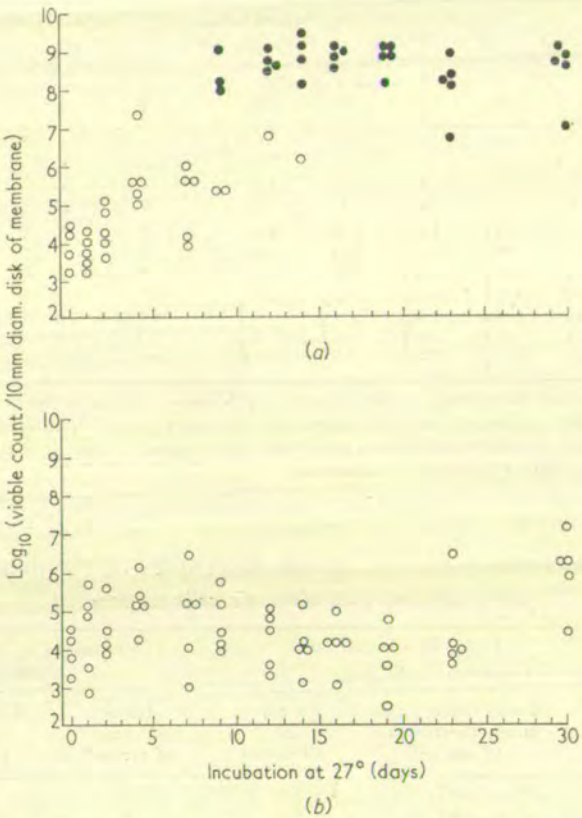

Fig. 1. The growth of *Ps. fluorescens* in the inner membrane of the air cell of eggs with (a) air cells uppermost and (b) air cells downwards. The eggs were inoculated on the third day following laying. Open circles, the results obtained with one egg; closed circles, fluorescent green pigment in the inner membrane of theair cell.

visible evidence of infection of the contents, and second, those in which there was a marked increase in the size of the population of the air cell membrane and gross contamination of the albumen. It will be seen from Table 2 that in the latter instance these changes were associated with the presence of a bacterial pigment in the inner membrane of the air cell and on the surface of the yolk. The pigment was always on, or in close proximity to, the blastoderm. It is known that the yolk, as a result of its gravimetric asymmetry, tends to reach a resting position in which the blastoderm is uppermost. The evidence suggests that the sudden increase in the

size of the populations in the inner membrane of the air cell results from contact of the yolk with the inoculated membrane.

TABLE 1

The recovery of viable organisms from the albumen of eggs the air cells of which had been inoculated with Ps. fluorescens

Time at 27° (days)	Viable count (log_{10}/ml of albumen) of organisms in eggs with Air cell uppermost, for egg no.					Air cell lowermost, for egg no.				
	1	2	3	4	5	1	2	3	4	5
1	*	*	*	*	*	*	*	*	*	*
2	*	*	*	*	*	2·7	*	*	*	*
4	*	*	*	†	2·2	*	*	*	*	*
7	*	*	*	*	*	*	*	*	*	2·5
9	‡	7·2§	3·0	6·0§	3·0	*	*	*	*	*
12	*	5·6	6·6	7·9	7·6	*	*	*	*	*
14	9·0§	8·7§	6·3§	1·4	8·8	*	*	*	*	*
16	8·5§	8·7§	9·1§	9·2§	9·0§	*	*	*	*	*
19	9·4§	8·6§	8·6§	8·4§	8·6§	2·2§	8·0	2·9	3·0	2·6
23	6·7§	8·4§	8·4§	9·0§	7·7§	1·3	2·8	2·5	2·9	6·1§
30	8·5§	8·7§	8·7§	8·0§	9·1§	**	*	**	*	3·0

* Viable organisms not recovered. † Not tested. ‡ Viable organisms were not recovered from the albumen of this egg but fluorescent pigment was observed in the membrane of the air cell. § Fluorescent green pigment was observed in the membrane of the air cell. ** More than 500 organisms occurred/ml of fluorescent albumen.

TABLE 2

Changes in the appearance of eggs inoculated with Ps. fluorescens *and incubated with their air cells uppermost*

Time at 27° (days)	Egg no.	Log_{10} viable bacteria in 10 mm diam. disk of inner membrane of air cell	Log_{10} viable bacteria in 1 ml of albumen	Presence† of fluorescent green pigment in Inner membrane of air cell	Surface of yolk	Albumen
8	1	8·7	4·3	F	F	—
	2	8·4	6·5	F	F	—
9	3	8·3	*	F	F	—
	4	8·3	7·0	F	F	F
	5	6·9	*	F	—	—
	6	8·3	2·0	F	F	—
	7	8·3	6·0	F	F	F
	8	8·6	7·0	F	F	F
10	9	8·6	6·6	F	—	F
	10	8·3	6·8	F	F	f
	11	8·5	>3·0	F	F	F

* Viable organisms not recovered. † Fluorescence under ultraviolet radiation: —, absent; F, occurring in patches; f, disseminated throughout the albumen.

Support for this inference was provided by the eggs in which the yolk moved away from the site of the inoculum. It was found (Fig. 1) that the initial increase in the numbers of organisms present in the inner membrane of the air cell was followed

by a phase (9–16 days) during which the size of the populations declined gradually. During this time, also, no contamination of the albumen was detected. In the period following this decline, extensive infection of the albumen occurred in some of the eggs and, in several of these, there was an increase in the number of organisms in the inner membrane of the air cell. The results obtained at this time are summarized in Table 3, from which it will be seen that gross contamination of the albumen was generally associated with the presence of the organism's pigment on the surface of the yolk, particularly that part which had made contact with the shell membranes.

The sequence of events discussed above was observed in eggs inoculated with the following organisms (the bracketed figures refer to the number of strains tested): *Alcaligenes faecalis* (1), a nonpigmented pseudomonad (1), *Ps. fluorescens* (7), *Aeromonas liquefaciens* (2), *Cloaca* sp. (1), *Serratia marcescens* (1) and *Salmonella* spp. (2). It would seem, therefore, that there are two phases of multiplication in the course of bacterial infection of the hen's egg.

TABLE 3

Changes in the appearance of eggs inoculated with Ps. fluorescens *and inoculated with their air cells downwards*

Time at 27° (days)	Egg no.	Log_{10} viable bacteria in		Presence† of fluorescent green pigment in		
		10 mm diam. disk of inner membrane of air cell	1 ml of albumen	Inner membrane of air cell	Surface of yolk	Albumen
19	1	4·0	2·2	–	F	F
	2	4·0	8·0	–	–	–
	3	4·8	2·9	–	–	–
	4	2·6	3·0	–	–	–
	5	3·7	2·6	–	–	–
23	6	3·9	1·3	–	–	–
	7	3·7	2·8	–	–	–
	8	3·9	2·4	–	–	–
	9	4·0	2·9	–	–	–
	10	6·5	6·1	–	F	F
30	11	6·3	‡	F	F	F
	12	4·4	*	–	–	–
	13	7·0	‡	–	F	–
	14	6·4	*	–		–
	15	5·9	3·0	–	–	–

* Viable organisms not recovered. † Fluorescence under ultraviolet radiation: –, absent; F, present. ‡ More than 500 viable organisms occurred/ml of albumen.

Primary phase of multiplication

The results shown in Table 1 and Fig. 1 indicate that the primary phase of multiplication was confined to the shell membranes, and that migrants from this source were unable to proliferate in the albumen. Additional information relating to the second observation was obtained from eggs which had received a large inoculum. The results in Table 4 were obtained with a fermentative organism, *Aer. liquefaciens* CF 10/1, but essentially the same results were obtained both with nonproteolytic

and a proteolytic strain of *Ps. fluorescens*. The use of a large inoculum resulted in gross contamination of the albumen within a short period following inoculation. However, significant changes in the pH or glucose concentration in the albumen were detected only in eggs in which there was definite evidence of a union of the yolk and the shell membrane. It appeared, therefore, that contaminants derived from the shell membrane required some substance(s) supplied by the yolk before they were able to utilize glucose, the most readily available energy source in the albumen.

The results presented in Fig. 2 indicate that the rate of multiplication in the primary phase was influenced by the medium used to suspend the inoculum. In Fig. 2 (*a*),

TABLE 4

Changes in the pH and glucose concentration in the albumen of eggs inoculated with Aer. liquefaciens

Time at 27° (days)	Egg no.*	Log_{10} viable organisms in: Inner membrane of air cell	Log_{10} viable organisms in: 1 ml of albumen	Macroscopic changes‡ in contents of egg	Albumen: pH	Albumen: Glucose (mg/ml)
3	1	7·4	5·5	−	9·3	2·43
	2	4·3	6·1	−	9·3	3·40
	3	7·4	5·9	−	9·3	3·07
	4	7·3	5·6	−	9·3	3·86
	5	†	†	−	9·4	2·05
	6	†	†	−	9·4	3·11
10	1	6·8	†	−	9·3	1·91
	2	5·2	†	−	9·3	1·63
	3	9·5	8·2	+	8·6	0·31
	5	†	†	−	9·2	2·00
	6	†	†	−	9·2	1·80
16	1	9·0	8·8	+	8·1	NT
	2	9·0	†	−	9·3	1·83
	3	9·0	7·0	+	8·7	0·22
	4	9·0	8·5	+	8·6	0·14
	5	†	†	−	9·6	1·98
	6	†	†	−	9·6	1·74

*Eggs no. 1–4 were inoculated with 0·1 ml of 18 h broth culture; eggs no. 5 and 6 were inoculated with 0·1 ml of sterile nutrient broth (controls). † Viable organisms not recovered. ‡ Macroscopic changes in the appearance of the egg: +, custard-like material present on that part of the yolk that had made contact with the shell membranes; −, none.

NT, not tested.

for example, the rate of multiplication of a nonpigmented pseudomonad applied as a suspension in distilled water was considerably less than when the organisms were suspended in an infusion of deep litter material (0·5 g of deep litter in 100 ml of water). Extraction with 0·5% (v/v) 8-hydroxyquinoline in chloroform diminished the influence of the infusion on the rate of multiplication but the addition of $FeSO_4.7H_2O$ to the extracted infusion to give a final concentration of 100 μg/ml resulted in a marked increase. Results similar to these were obtained with a strain of *Ps. fluorescens* and it will be seen from Fig. 2 (*b*) that the addition of $FeSO_4$ to distilled water resulted in multiplication at a rate similar to that obtained when the inoculum was suspended in an infusion of deep litter containing $FeSO_4$. It would appear,

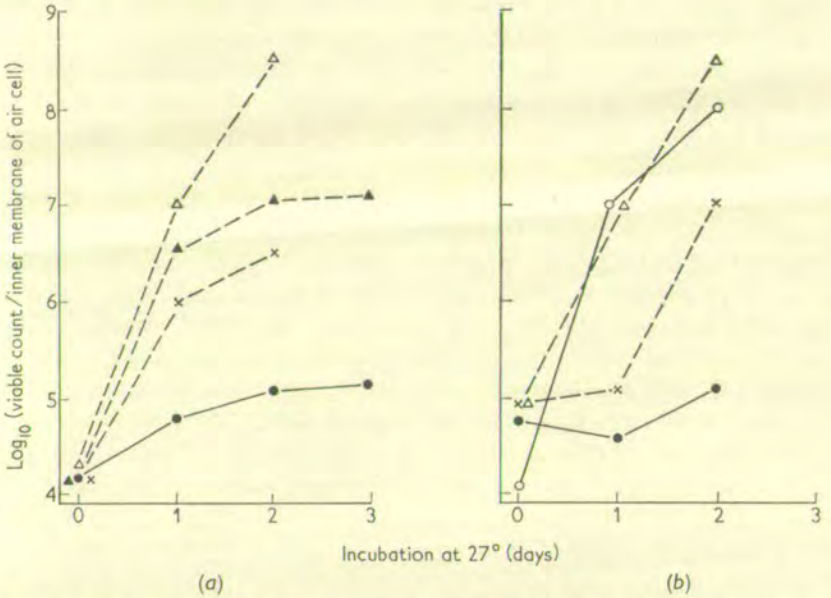

Fig. 2. The influence of various suspending media on the rate of multiplication of a nonpigmented pseudomonad (a) and *Ps. fluorescens* (b) in the inner membrane of the air cell of eggs. Eggs were inoculated with: distilled water (closed circles); distilled water containing $FeSO_4.7H_2O$ (100 μg/ml) (open circles); an infusion of deep litter (closed triangles); an extracted infusion of deep litter containing $FeSO_4.7H_2O$ (100 μg/ml) (open triangles); infusion of deep litter extracted with 8-hydroxyquinoline (crosses).

therefore, that the rate and the extent of bacterial multiplication during the primary phase was controlled by the nature and availability of the nutrients present in the extraneous materials deposited on the shell membranes along with the organisms.

When the organisms were suspended in distilled water, the primary phase of multiplication ended on the 3–4th day following inoculation. This occurred presumably when the organisms had consumed the nutrients present in the shell membranes and within themselves. Thereafter, there was a gradual decline in the size of the populations in the inner membrane of the air cell but no detectable increase in the contamination of the albumen. During this phase, the formation of macroscopic colonies on nutrient agar by some of the strains of bacteria required 48 h incubation whereas colonies of comparable size were formed within 24 h incubation when the organisms were isolated during the primary or secondary phase of multiplication.

Secondary phase of multiplication

In the experiments discussed earlier, the results indicated that the second phase of multiplication began when the yolk made contact with the shell membranes. It will be seen from Table 2 that a greater than average number of pseudomonads in a membrane tinted with the organism's pigment was not always associated with either gross or detectable contamination of the albumen. Thus it would seem that this

phase began in the shell membranes and continued in the contents of the egg at which time the organisms utilized the glucose present in the albumen. The formation of an albumen-free nidus between the yolk and the shell membranes or the provision of nutrients by the yolk are possible interpretations of these observations. The induction of renewed multiplication by the latter method was attempted in the following experiments.

It has been shown (Schade & Caroline, 1944) that the chelation of iron by the protein, conalbumin, prevents the growth of micro-organisms in egg albumen. It would appear that this substance plays a primary role in retarding the manifestation of rotting in commercial eggs that have been contaminated with the commonly occurring rot-producing bacteria (Brooks, 1960; Garibaldi, 1960). Preliminary investigations showed that all the organisms used in the present study would proliferate in albumen supplemented with $FeSO_4$ but not in albumen supplemented with sulphates of other metals. The influence of $FeSO_4$ on bacterial multiplication in the intact egg was examined in the following manner. A suspension of washed bacteria was inoculated into the air cell and the eggs, with their air cells downwards,

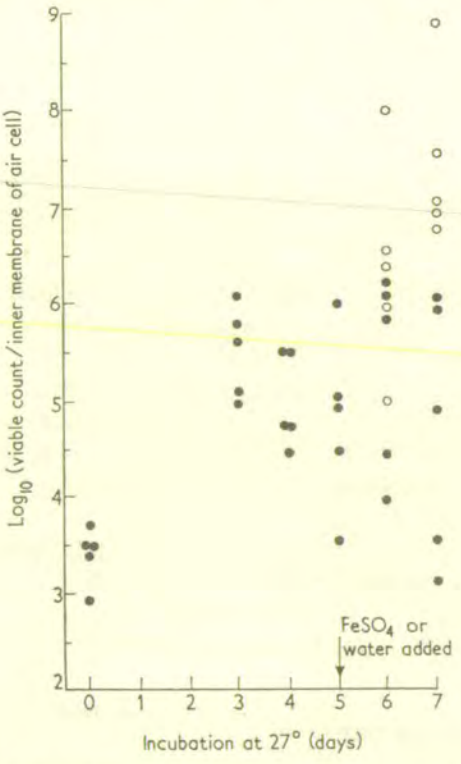

Fig. 3. The effect of the addition of $FeSO_4$ to the albumen on the multiplication of a nonpigmented pseudomonad in the inner membrane of the air cell. Closed circles, normal eggs and eggs injected (into the albumen) with 0·1 ml of water after the 5th day of incubation. Open circles, eggs injected (into the albumen) with 0·1 ml of 2·5% (w/v) $FeSO_4.7H_2O$.

were incubated at 27°. On the 5th day following inoculation, a hole was drilled in the shell very near the air cell and 0·1 ml of a 2·5% (w/v) aqueous solution of $FeSO_4.7H_2O$, or in the case of controls, 0·1 ml water was placed in the albumen.

The $FeSO_4$ became disseminated through the thin white in the 24 h following inoculation and through the albuminous sac in the next 24 h. The populations in the inner membrane of the air cell were in the phase of decline when the $FeSO_4$ was added (Fig. 3). It will be seen from Fig. 3 that the addition of $FeSO_4$ caused a renewal of multiplication whereas the addition of water did not have any detectable influence on the populations. The results summarized in Table 5 show that a similar response was given by a number of different types of Gram-negative bacteria.

TABLE 5

*The influence of iron added to the albumen on bacterial multiplication in the inner membrane of the air cell**

Organism	Strain	Log_{10} viable organisms/inner membrane of air cell of eggs injected with†	
		Distilled water	Solution of iron
A. faecalis	G21/3	6·0	8·0
	G32/1	4·8	8·4
Nonpigmented pseudomonad	G8/1	6·5	9·3
	G11/1	5·6	9·0
Ps. fluorescens	CF21	4·7	8·6
	G10	3·8	8·0
Proteus vulgaris	G14	4·4	8·0
	G16	6·8	8·3
Cloaca sp.	G75/2	3·2	7·7
Aer. liquefaciens	CF17/1	6·8	8·9

* The inoculated eggs were incubated for 5 days at 27° before their albumens were injected with iron or distilled water; the air cells were downwards throughout incubation. † Colony counts were made on the inner membrane of the air cell on the 2nd day following the addition to the albumen of 0·1 ml of distilled water or 0·1 ml of 2·5% (w/v) $FeSO_4.7H_2O$; the counts given are the average obtained from the membranes of 3 eggs.

Changes in eggs produced by different types of bacteria

When the numbers of viable organisms in the inner membrane of the air cell and the albumen was used to follow the course of bacterial infection of the egg, the sequence of events shown in Fig. 1 and Table 1 was common to all the organisms examined. However, the macroscopic changes in the contents of the egg varied with different types of bacteria. After prolonged incubation (42 days at 27°) these changes were characteristic for particular types of bacteria. In order to augment the data obtained from the experiments discussed earlier, several Gram-negative bacteria were inoculated separately into eggs. Four eggs were inoculated with a single organism and the changes in the contents were recorded after incubation at 27° for 42 days. In addition the purity of the infection was checked by subculturing a loopful of

TABLE 6

Changes in eggs produced in 42 days at 27° by different bacterial species and a presumptive correlation of the salient features of the rots with the metabolic activities of the causative organism

Organism	No. of strains tested	Metabolic activity*				Salient features of rot	Type of rot‡
		Proteolytic	H_2S produced	Lecithinase produced	Pigment produced		
Aer. liquefaciens	8	++	++	+††	−	Gelatinous yolk blackened throughout; grey watery albumen	Black rot type 1§
Proteus vulgaris	4	++	++	−	−	Dark brown mealy yolk; dark brown albumen	Black rot type 2§
Serratia marcescens	1	†	†	+	+	Red discoloration of albumen and surface of yolk; yolk encrusted with custard-like material	Red rot
Ps. fluorescens	5	+	−	−	+	Mealy yolk; fluorescent green albumen	Pink rot**
	4	−	−	−	+	Fluorescent green pigment in albumen	Fluorescent green rot**
Nonpigmented pseudomonad	4	+	+	−	−	Gelatinous amber-like yolk striped with olive green pigment; almond-like odour	Green rot**
Cloaca sp.	6	+††	+††	+	−	Yolk encrusted with custard-like material and occasionally flecked with olive-green pigment	Custard rot**
	8	−	−	−	−		
Salmonella spp.	2	−	−	−	−	Occasional faint turbidity in the albumen	Colourless rot
Citrobacter sp.	7	−	−	−	−		
Alcaligenes faecalis	4	−	−	−	−		

* Activity: −, negative; +, strong; ++, very strong. † Not tested.

‡ The rots were identified according to the descriptions given by Florian & Trussell (1957)§ or by Haines (1939).**

†† Although positive, it was concluded that this property did not play a significant role in the rotting of eggs.

the albumen on nutrient agar. The results are summarized in Table 6. The changes occurring in eggs divided the organisms into 2 major groups. In one the contents of the eggs manifested profound changes the salient features of which appeared to be related to the metabolic activities of the causative organism. In general terms the organisms possessed one or more of the following properties: chromogenesis, proteolytic activity, ability to produce H_2S from heat-coagulated egg white or the ability to attack lecithin. In some instances the extent of these changes appeared to be correlated with the relative activity of a particular attribute. For example, strains of *Aer. liquefaciens* were actively proteolytic, as judged by the speed of liquefaction of nutrient gelatin, and they produced H_2S at an early stage in their growth in peptone water containing heat-coagulated egg white. These organisms digested the albumen, caused gelation of the yolk and produced blackening throughout the entire yolk. A nonpigmented pseudomonad, on the other hand, was weakly proteolytic but produced easily detectable amounts of H_2S. This organism did not digest the albumen but it did produce gelation of the yolk, the periphery of which was flecked olive-green, a discoloration which could be removed by H_2O_2. The organisms of the second group did not have any of the properties of the first group. The course of infection in eggs inoculated with these organisms was the same as that shown in Fig. 1 and Table 1 but, apart from an occasional faint turbidity in the albumen, eggs inoculated with these organisms could not be disintguished from uninoculated eggs which had been incubated for a similar period of time.

Discussion

Gillespie & Scott (1950), using information derived mainly from empirical observations, considered the following to be possible stages in the infection of an egg: first, contamination and penetration of the shell; second, colonization and penetration of the shell membranes; and third, infection of the contents. A number of investigators have noted a lag of 10–20 days following bacterial penetration of the shell and before the recovery of viable organisms from or the detection of changes in the contents of the eggs (Zagaevsky & Lutikova, 1944; Gillespie & Scott, 1950; Bigland & Papas, 1953; Stokes *et al.*, 1956; Orel, 1959; Fromm & Monroe, 1960; Garibaldi & Bayne, 1960). It is known that the shell membranes can act as bacterial filters (Haines & Moran, 1940; Garibaldi & Stokes, 1958) but that this property is lost during contact of the membranes with bacteria (Walden, Allen & Trussell, 1956; Garibaldi & Stokes, 1958). The gross contamination of the albumen within a short period following the inoculation of the air cell with a heavy suspension of bacteria (Table 4) did suggest that the filter-like property of the shell membranes was not primarily responsible for the confinement of the infection that was noted in eggs the air cells of which had been seeded with about 1,000 organisms. In fact, the low level of contamination of the albumen in the latter instance, together with the indirect evidence that contaminants were unable to proliferate in the albumen, did suggest that certain properties of the albumen were mainly responsible for confining the infection to the shell membranes.

The antimicrobial nature of the albumen of the hen's egg has long been recognized. In the course of time the following components have been postulated as a result of

experiments conducted during the investigation of problems that were not directly concerned with the microbiology of the egg: the lytic and flocculating action of lysozyme (Laschtschenko, 1909; Salton, 1957); the alkaline reaction of the albumen (Sharp & Whitaker, 1927); the combination of biotin with avidin (Eakin, Snell & Williams, 1940); the combination of riboflavin with an uncharacterized protein (Rhodes, Bennett & Feeney, 1959); the chelation of iron by conalbumin (Schade & Caroline, 1944) and the low content of nonprotein nitrogen (Haines, 1939). Consequently, although there is much circumstantial evidence concerning the individual components of this defence, there is neither direct evidence relating to their co-ordination in the whole egg nor information that can be used to assess their relative importance. In view of the renewed multiplication that attended the addition of $FeSO_4$ to the albumen, it would appear that, with eggs contaminated with the commonly occurring rot-producing bacteria, the chelation of iron by conalbumin was most probably responsible for confining the infection to the shell membranes.

Implications of commercial importance attend the observation that multiplication in the primary phase was influenced by the nature of the substances deposited on the shell membranes along with the organisms. The organizers of egg marketing in many countries have attempted to dissuade producers from washing eggs because it was considered that the unpredictable storage characteristic of washed eggs was attributable to bacterial penetration of the shell at the time of cleaning. Some recent observations have shown that the rate and incidence of rotting can be increased by washing eggs in water contaminated, either naturally or artificially, with iron (Garibaldi & Bayne, 1962*a*,*b*). It would appear from the results obtained in the present investigation that this may be due to an increase in the rate and extent of bacterial multiplication in the shell membranes.

The evidence obtained in the present study indicated that a second phase of bacterial multiplication occurred and, with certain organisms, symptoms of rotting developed when the yolk made contact with the shell membranes. It is well known that the antimicrobial properties of the albumen are destroyed by substances present in the yolk and Zagaevsky & Lutikova (1944) noted rapid rotting in eggs in which the membranes surrounding the yolk were ruptured deliberately. Thus it would appear that effective bacterial infection is confined to the shell membranes until the yolk makes contact with these structures. This could account for the lag noted by the workers who were cited at the beginning of the Discussion. The rate of deterioration of the albuminous sac, and thus the rate of rising of the yolk, is influenced by many factors of which the initial quality of the thick white and the temperature of storage are of prime importance. In consequence, the physical properties of the albumen may play a role in the antimicrobial defence of the egg.

Many investigators have found that rotten eggs contain a mixed population of Gram-negative bacteria (Miles & Halnan, 1937; Haines, 1939; Richard & Mohler, 1950; Florian & Trussell, 1957). Since the report of Miles & Halnan (1937) it has become customary to inoculate eggs with pure cultures in order to establish the identity of the organism that was responsible for the changes observed in the original rot. The other organisms have been ignored or referred to as 'secondary invaders' (e.g. Florian & Trussell, 1957). The present investigation has revealed that the

pattern of multiplication of the latter class was essentially the same as that of the rot-producing bacteria. They differ from the last mentioned in not possessing the following attributes: chromogenesis, proteolytic activity, ability to produce H_2S or attack lecithin. It would seem that no useful purpose is served by designating the isolates as primary or secondary invaders. A utilitarian classification, based on the data presented in Table 6, could be achieved if stress was given to the salient features of the rot and appending to this the name of the causative organism. In ecological terms, this organism could be regarded as the 'dominant' species, a term that need not imply numerical preponderance.

Egg products, such as frozen whole egg, egg powder, etc. can contain many viable organisms ($1–10 \times 10^5$/g; Brooks & Taylor, 1955) but the relative importance of many possible sources of contamination have not been determined. In view of the observation that eggs infected with nonpigmented, nonproteolytic organisms did not exhibit easily detected signs of infection, it could be expected that this type of egg would not be recognized by the 'look and smell' test that is used routinely in egg breaking plants. It is noteworthy that Johns & Bérard (1945, 1946) found several such eggs during an investigation of sources of contamination in an egg breaking plant. The finding that salmonellae can proliferate in eggs without producing symptoms other than a faint turbidity of the albumen is in accord with the observations of Stokes *et al.* (1956). In commerce, such eggs could be an important and, until now, unsuspected source of contamination of egg products.

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References

Bigland, C. H. & Papas, G. (1953). Experiment in egg penetration by salmonella. *Canad. J. Comp. Med.* **17**, 105.

Brooks, J. (1960). Mechanism of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.* **23**, 499.

Brooks, J. & Taylor, D. J. (1955). Eggs and egg products. *Rep. Fd Invest. Bd, Lond.* no. 60. London: H.M.S.O.

Eakin, R. E., Snell, E. E. & Williams, R. J. (1940). A constituent of raw egg white capable of inactivating biotin *in vitro*. *J. biol. Chem.* **136**, 801.

Elliott, R. P. (1954). Spoilage of shell eggs by pseudomonads. *Appl. Microbiol.* **2**, 158.

Florian, M. L. E. & Trussell, P. C. (1957). Bacterial spoilage of shell eggs. IV. Identification of spoilage organisms. *Food Tech., Champaign* **11**, 56.

Fromm, D. & Monroe, R. J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg shell permeability. *Food Tech., Champaign* **14**, 401.

Garibaldi, J. A. (1960). Factors in egg white which control growth of bacteria. *Food Res.* **25**, 337.

Garibaldi, J. A. & Bayne, H. G. (1960). The effect of iron on pseudomonas spoilage of experimentally infected shell eggs. *Poult. Sci.* **39**, 1517.

Garibaldi, J. A. & Bayne, H. G. (1962*a*). Iron and the bacterial spoilage of shell eggs. *J. Fd Sci.* **27**, 57.

Garibaldi, J. A. & Bayne, H. G. (1962*b*). The effect of iron on *Pseudomonas* spoilage of farm washed eggs. *Poult. Sci.* **41**, 850.

Garibaldi, J. A. & Stokes, J. L. (1958). Protective role of shell membranes in bacterial spoilage of eggs. *Food Res.* **23**, 283.

Gillespie, J. M. & Scott, W. J. (1950). Studies in the preservation of shell eggs. IV. Experiments on the mode of infection by bacteria. *Aust. J. appl. Sci.* **1**, 514.

Haines, R. B. (1939). Microbiology in the preservation of the hen's egg. *Rep. Fd Invest. Bd, Lond.*, no. 47. London: H.M.S.O.

Haines, R. B. & Moran, T. (1940). Porosity of, and bacterial invasion through, the shell of the hen's egg. *J. Hyg., Camb.* **40**, 453.

Johns, C. K. & Bérard, H. L. (1945). Further bacteriological studies relating to egg drying. *Sci. Agric.* **24**, 551.

Johns, C. K. & Bérard, H. L. (1946). Effect of certain methods of handling upon the bacterial content of dirty eggs. *Sci. Agric.* **26**, 11.

Laschtschenko, P. (1909). Über die keimtötende und entwicklungshemmende Wirkung Hünereiweiss. *Z. Hyg. Infektionskrankh.* **64**, 419.

Miles, A. A. & Halnan, E. T. (1937). A new species of micro-organism (*Proteus melanovogenes*) causing black rot in eggs. *J. Hyg., Camb.* **37**, 79.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 752.

Orel, V. (1959). The *Pseudomonas* spoilage of eggs laid by individual hens. *Poult. Sci.* **38**, 8.

Rhodes, M. B., Bennett, N. & Feeney, R. E. (1959). The flavoprotein-apoprotein system of egg white. *J. biol. Chem.* **234**, 2054.

Richard, O. & Mohler, A. (1950). Die Mikrobiologie der "Heu-eier". *Mitt. Lebensm. Hyg., Bern.* **41**, 168.

Salton, M. R. J. (1957). The properties of lysozyme and its action on micro-organisms. *Bact. Rev.* **21**, 82.

Schade, A. L. & Caroline, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science* **100**, 14

Sharp, P F. & Whitaker, R. (1927). The relation of hydrogen ion concentration of egg white to its germicidal action. *J. Bact.* **14**, 17.

Somogyi, M. (1945). A new reagent for the determination of sugars. *J. biol. Chem.* **160**, 61.

Stokes, J. L., Osborne, W. W. & Bayne, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Food Res.* **21**, 510.

Waldon, C. C., Allen, I. V. F. & Trussell, P. C. (1956). The role of the egg shell and shell membranes in restraining the entry of micro-organisms. *Poult. Sci.* **35**, 1190.

Zagaevsky, J. S. & Lutikova, P. O. (1944). Sanitary measures in the egg breaking plant. *U.S. Egg Poult. Mag.* **50**, 17.

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Influence of Temperature on Bacterial Infection of the Hen's Egg[1]

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ABSTRACT

BOARD, R. G. (Iowa State University, Ames), AND J. C. AYRES. Influence of temperature on bacterial infection of the hen's egg. Appl. Microbiol. **13**:358–364. 1965.—Temperature of incubation had a marked effect on infection of eggs in which the air cells had been inoculated with a washed suspension of *Serratia marcescens*. There was no evidence of bacterial multiplication or spoilage in eggs held at 10 C for 42 days. Multiplication occurred in the shell membranes of eggs held at 30 or at 37 C when the yolk made contact with these membranes, and continued in the contents of the egg, at which time the first signs of spoilage appeared. In a few eggs, very large populations were present in the shell membranes and in the albumen. In eggs inoculated with *Pseudomonas fluorescens* and held at 10 C, bacterial multiplication occurred in the shell membranes in the first 7 days of incubation. These populations did not appear to change in size in the 7- to 14-day period of incubation. Renewed multiplication and concomitant spoilage of the contents was observed in many of the eggs thereafter.

When eggs are held in the temperature range of 15 to 30 C, there is a lag of 10 to 20 days after bacterial penetration of the shell and before the recovery of appreciable numbers of viable organisms from or the development of macroscopic changes in the contents (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Stokes, Osborne, and Bayne, 1956; Orel, 1959; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960). Recent observations (Brooks, 1960; Board, 1964) indicate that, because of the antimicrobial defense of the albumen, bacterial multiplication is confined to the shell membranes during this period and that this confinement persists until the yolk makes contact with the shell membranes. The work to be described was undertaken with the objective of determining whether or not the temperature of incubation influenced the duration of this lag.

MATERIAL AND METHODS

Organisms. Serratia marcescens 2G12 was used in the main part of this work, but five strains of *Pseudomonas fluorescens* were used for comparative purposes. The last-mentioned organisms had been isolated from rotten eggs or chicken carcasses. Stock cultures in nutrient broth (Difco) were stored in a domestic refrigerator at 4 C, and, for experimental purposes, an organism was subcultured on three occasions immediately before use. All cultures were grown in nutrient broth at 30 C, and after 18 hr the cells were harvested by centrifugation, twice washed in 0.067 M Sørensen's phosphate buffer (*p*H 7.2), and resuspended finally in distilled water.

Eggs. Eggs were produced by an unmated flock of White Leghorns and stored at 10 C for 4 days prior to inoculation. All eggs were candled, and those of poor internal quality were not used. The eggs weighed between 50 and 60 g.

Inoculating and sampling. The method has been fully described elsewhere (Board, 1964). Briefly, 0.1 ml of an inoculum containing about 10^4 organisms per milliliter was injected into the air cell of eggs, and the latter, with the air cell either uppermost or downwards, were incubated at 10, 30, or 37 C. At frequent intervals, five eggs were randomly selected, and the numbers of viable organisms were determined (i) in the inner membrane of the air cell, by placing 0.02 ml of dilutions prepared from the comminuted membrane on nutrient agar, and (ii) in the albumen, by preparing nutrient agar pour plates containing 1 ml of appropriate dilutions.

RESULTS

In preliminary experiments, attention was given to the influence of temperature and media on the growth of *S. marcescens* 2G12. Good growth occurred on slopes of nutrient agar (Difco) incubated at 10, 30, or 37 C. The results

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obtained with albumen or a mixture of yolk (1 volume) and albumen (1 volume) are summarized in Fig. 1. Extensive bacterial multiplication took place in the mixture during 48 hr of incubation at all three temperatures. With albumen alone, there was a notable reduction in the number of viable organisms during incubation at 10 or 37 C but not at 30 C. The multiplication which occurred in the latter instance was negligible when compared with that in the mixture. Results similar to these were obtained by Ayres and Taylor (1956), who studied bacterial growth in both the intact and the broken-out egg. It is pertinent, also, to note the results of Sharp and Whitaker (1927). These workers observed a rapid decline in the number of viable organisms in albumen inoculated with young broth cultures of gram-negative bacteria incubated at 37 C. This decline did not occur when thermally denatured albumen adjusted to *p*H 9.4 was used. This evidence indicated that the ability of gram-negative bacteria to survive in an unfavorable environment of egg albumen may be influenced by temperature.

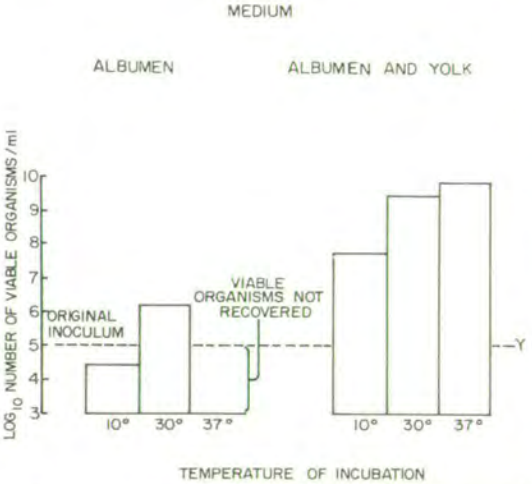

FIG. 1. *Influence of temperature on the growth during 48 hr of Serratia marcescens 2G12 in albumen alone and a mixture of yolk (one volume) and albumen (one volume). Viable organisms not recovered at 37 C; y, size of the original inoculum.*

The changes occurring in the population of *S. marcescens* in the inner membrane of the air cell of eggs held, with their air cells uppermost, at 10, 30, or 37 C are summarized in Fig. 2. The number of viable organisms in the inner membrane of eggs held in a refrigerated (10 C) display cabinet did not change appreciably during the 42-day period of observation. Viable organisms were rarely recovered from 1 ml of albumen, and the eggs did not manifest signs of spoilage.

A different sequence of events was obtained with eggs held at 30 C in a bacteriological incubator. A slight increase in the number of viable organisms was noted in the 5 days after inoculation, and very large populations were present both in the shell membranes and the albumen on and after the 10th day of incubation (i.e., when the eggs were 14 days of age). This gross contamination was observed at a time when it was noted that the yolk was making contact with the inoculated inner membrane of the air cell. Evi-

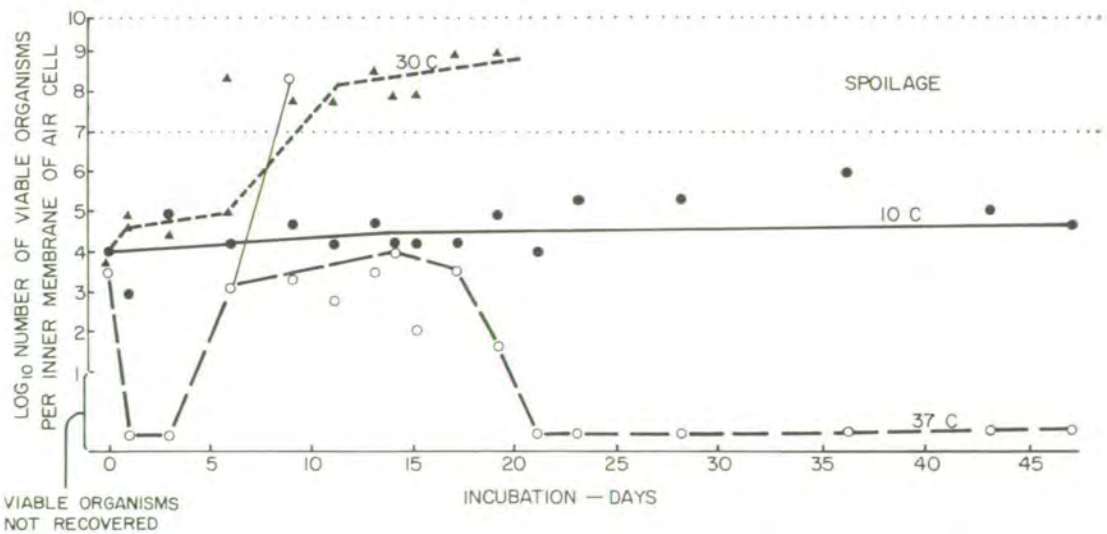

FIG. 2. *Changes in the size of the populations of Serratia marcescens 2G12 in the inner membrane of the air cell of eggs held at 10, 30, or 37 C. Each point is the average obtained from five eggs.*

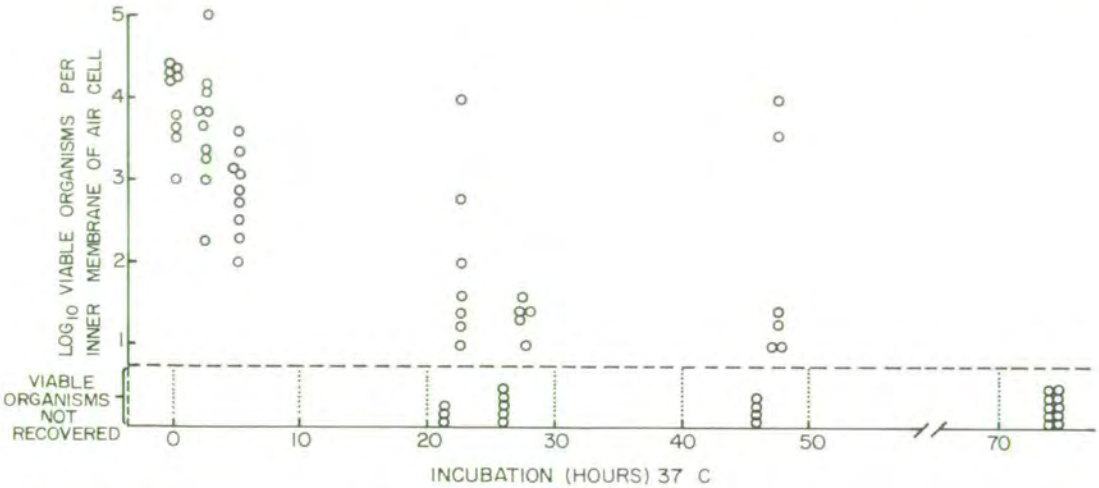

FIG. 3. *Changes in the populations of Serratia marcescens in the inner membrane of the air cell of eggs held at 37 C.*

dence of this union was obtained during candling of the eggs as well as from inspection of the broken-out eggs. In the latter instance, the pigment produced by *S. marcescens* was present on the inner membrane of the air cell and on the surface of the yolk at the place of their union. On further incubation, the pigment became disseminated throughout the white and over the entire surface of the yolk. This sequence of events is essentially the same as those described elsewhere (Brooks, 1960; Board, 1964), and it was concluded that a secondary phase of bacterial multiplication, together with manifestations of spoilage, was induced by the union of the yolk and the inoculated shell membranes.

Yet another sequence of events was observed in eggs held at 37 C. There was a sharp decline (Fig. 2) in the number of viable organisms in the inner membrane of the air cell in the 2 days following inoculation. The data summarized in Fig. 3 and 4 indicate that this was due to migration of organisms from the membranes and the death of the migrants in the albumen. Thus, on the 2nd day after inoculation, it was not possible to recover viable organisms from either the shell membranes or the contents of the majority of eggs, and this situation prevailed until the 5th to 7th day of incubation. At this time, the yolk made contact with the inoculated membrane of the air cell, and this was associated with the recovery of viable organisms from the shell membranes and, to a lesser extent, from the albumen. In exceptional instances, populations of a million or more viable organisms were found in the inner membranes of the air cell as well as in 1 ml of the albumen, and the contents of the eggs were stained throughout with pigment produced

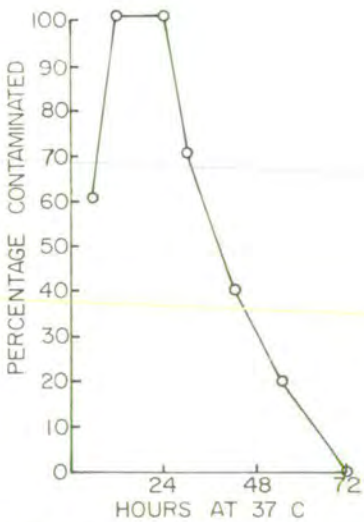

FIG. 4. *Incidence of contamination of the albumen of eggs, incubated at 37 C, in which the air cell had been inoculated with Serratia marcescens. Ten eggs were examined on each occasion.*

by *S. marcescens*. In the majority of eggs, however, populations in the inner membrane of the air cell reached a size of 100 to 10,000 viable organisms, but very few organisms were recovered from the albumen. This level of contamination was a regular feature of all the eggs examined after 1 to 3 weeks after inoculation, yet none of the eggs exhibited signs of spoilage. After this period, viable organisms were not recovered from the shell membranes or from the albumen.

These results confirm observations of Wolk,

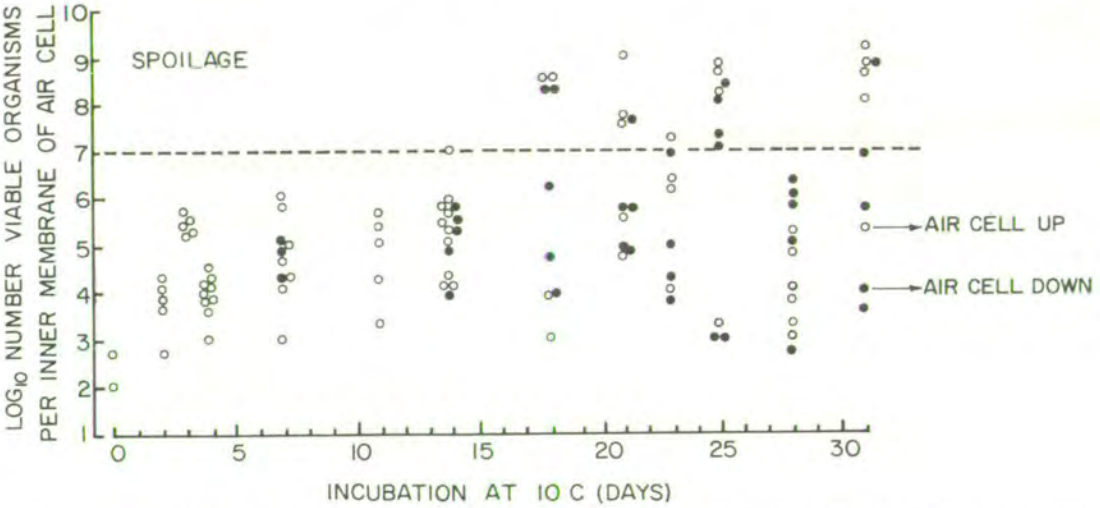

FIG. 5. *Changes in the size of the populations of Pseudomonas fluorescens No. 8 in the inner membrane of the air cell of eggs held at 10 C. Results obtained from experiments 11 and 12 have been combined.*

McNally, and Spicknall (1950) and of Ayres and Taylor (1956) that temperature influences the course of bacterial infection of the hen's egg. Further, the effect of temperature has been noted in eggs infected with *Aeromonas liquefaciens* (Miles and Halnan, 1937), *Proteus vulgaris* (Board, 1962), and other common contaminants of rotten eggs (Ayres and Taylor, 1956; Board et al., 1964). The results obtained with eggs held at 10 C are of particular importance because of the widespread use of refrigerated display cabinets by retailers of eggs. It was for this reason that several strains of *Pseudomonas fluorescens* were used in an extensive investigation of the course of infection in eggs stored at this temperature.

The results given in Fig. 5 and Table 1 were obtained with *P. fluorescens* No. 8, but essentially the same results were given by four other strains of this species. The eggs were inoculated with 100 to 1,000 organisms, and it will be seen from Fig. 5 that the number of viable organisms in the inner membrane of the air cells of the majority of eggs had increased to 10^4 to 10^6 by the 7th day of incubation and that the albumen of many of the eggs (Table 1) contained viable organisms. This level of contamination was found in eggs examined on the 14th day after inoculation with but one exception. In the latter instance, the membranes of the air cell contained 10^7 viable organisms, and the albumen, which was colored with the pigment produced by *P. fluorescens*, had 10^9 viable organisms per milliliter. The incidence of spoiled eggs increased during further incubation, but, at the same time, a decline in the levels of contamination was noted in eggs which did not manifest signs of infection. These trends were found in eggs held with their air cells uppermost as well as in those held in the reverse position. No explanation can be offered to account for the development of large microbial populations and symptoms of spoilage. It has been reported (Board, 1964) that the development of gross contamination and spoilage of the contents

TABLE 1. *Recovery of viable organisms from the albumen of eggs in which the air cells had been inoculated with Pseudomonas fluorescens No. 8**

Eggs held at 10 C	Eggs held with their air cells	Log_{10} number of viable organisms recovered from 1 ml of albumen of egg no.				
		1†	2	3	4	5
days						
2	Uppermost	−	+	+	−	−
3	Uppermost	−	−	−	+	+
4	Uppermost	+	+	+	−	+
7	Uppermost	+	+	−	NT	NT
	Downwards	+	+	+	NT	NT
14	Uppermost	+	+	−	9.9‡	−
	Downwards	−	−	−	−	−
21	Uppermost	7.8‡	7.2‡	7.2‡	3.4	1.4
	Downwards	8.6‡	−	−	−	8.2‡
28	Uppermost	−	−	+	+	+
	Downwards	+	+	−	−	−
31	Uppermost	2.7‡	−‡	3.3‡	8.2‡	−
	Downwards	6.9‡	2.9	2.5	3.5‡	8.5‡

* These results were obtained from experiment no. 11 (of Fig. 5).

† Symbols: + = fewer than 30 organisms present in 1 ml of albumen; − = no viable organisms recovered; NT = not tested.

‡ The pigment produced by the organism was present in the inner membrane of the air cell or albumen.

of eggs held at 27 C was due to a union of the yolk and the shell membranes; essentially the same phenomenon (Fig. 2) was noted in eggs inoculated with *S. marcescens* and held at 30 or 37 C. In eggs inoculated with *P. fluorescens* and held at 10 C, there was no evidence of a union of the yolk and shell membranes. The only feature of the results in marked contrast to those obtained with *S. marcescens* or those reported elsewhere (Board, 1964) was the early and persistent contamination of the albumen of the majority of eggs.

DISCUSSION

The results give additional support to the concept (Gillespie and Scott, 1950) that there are two distinct phases in the course of infection after bacterial penetration of the shell of the hen's egg. During the first phase, bacterial multiplication of a limited extent is confined to the shell membranes, and migrants from this source do not become established in the albumen (Brooks, 1960; Board, 1964). This confinement can last for 10 to 20 days in eggs held at 15 to 30 C (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Stokes, Osborne, and Bayne, 1956; Orel, 1959; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960), provided that iron has not been included with the inoculum (Brant and Starr, 1962; Garibaldi and Bayne, 1962*a*, *b*). The confinement is terminated when the yolk makes contact with the shell membranes; this induces a second phase of bacterial multiplication in the contents of the eggs, and, when chromogenic and proteolytic organisms are present, the first signs of spoilage appear (Board, 1964).

In view of the evidence obtained in the present investigation and that presented elsewhere (Sharp and Whitaker, 1927; Ayres and Taylor, 1956; Garibaldi, 1960), temperature appears to influence the pattern just described in three ways. It influences (i) the rate of bacterial multiplication during the phase in which this is confined to the shell membranes, (ii) the coordinated workings of the various components of the antimicrobial defense of the albumen, and (iii) the onset of the secondary phase of bacterial multiplication by virtue of the effect it has on the rate of deterioration of the internal quality of the egg.

Although it has been shown repeatedly that common contaminants of rotten eggs can multiply in a mineral salts solution containing shell membranes (Stuart and McNally, 1943; Stokes and Osborne, 1956; Elliott and Brant, 1957; Garibaldi and Stokes, 1958), recent observations (Board, J. Appl. Bacteriol, *in press*) suggest that the membranes are not a rich source of readily available bacterial nutrients. This view has received support from the observations (Board, 1964) that the rate and extent of bacterial multiplication in the shell membranes in situ is influenced greatly by the nature of the substances present in the extraneous materials deposited on the membranes along with organisms. The present investigation has shown that the behavior of microorganisms on the shell membranes in situ is influenced also by temperature and by inherent properties of an organism. *P. fluorescens*, well known for its ability to grow at low temperatures, multiplied in the membranes of eggs held at 10 C, whereas *S. marcescens* did not, even though it had been demonstrated (Fig. 1) that the strain used in the present investigation would grow in a yolk-albumen mixture at this temperature. This is perhaps another example of the well known phenomenon of one condition (i.e., the lack of nutrients in the membranes) assuming importance when another (temperature) is approaching the limits of tolerance of an organism.

The antimicrobial defense of the albumen has long been recognized, and many components have been described, mainly as the result of investigations not directly concerned with the microbiology of eggs. The following unsatisfactory features can be listed when the albumen is considered as a medium for microbial growth: the lytic and flocculating action of lysozyme (Laschtschenko, 1909; Salton, 1957), the alkaline reaction (*p*H 9.6) of the albumen (Healy and Peter, 1925; Sharp and Whitaker, 1927), the antitryptic activity of ovomucoid (Balls and Swenson, 1934; Lineweaver and Murray, 1947), the combination of biotin with avidin (Eakin, Snell, and Williams, 1940), the combination of riboflavine with an uncharacterized protein (Rhodes, Bennett, and Feeney, 1959), the chelation of iron by conalbumin (Schade and Caroline, 1944; Feeney and Nagy, 1952; Garibaldi, 1960), the low content of nonprotein nitrogen (Haines, 1939), and the contributing inhibitions of other factors (Matsushima, 1958; Rhodes, Bennett, and Feeney, 1960). Such an interpretation as this has received wide acceptance by egg microbiologists, although its implications have not been tested experimentally except in the case of conalbumin and its role in retarding the manifestations of spoilage in eggs infected with certain common contaminants of rotten eggs (Garibaldi, 1960; Garibaldi and Bayne, 1962*a*, *b*).

As yet, however, attention has not been given to the influence of temperature on the coordinated workings of the factors just listed, although there is evidence of a general nature (Sharp and

Whitaker, 1927; Ayres and Taylor, 1956) which suggests that it may play an important role. This supposition is supported by the results obtained in the present investigation. It would appear that this facet of egg microbiology is worthy of further exploration, and it is to be hoped that such investigation would link the interests of those who are concerned with the egg as an item of food with those who are interested in the immunity of the chicken embryo during early development.

Much is known concerning the deleterious effects of high storage temperatures on the internal quality of eggs (Wesley and Stadelman, 1959), but only recently has it been suggested (Board, 1964) that there is an interrelationship between the rate of breakdown of the albuminous sac and the rate of development of macroscopic changes in eggs infected with chromogenic or proteolytic bacteria. Further evidence of this relationship was obtained in the present investigation. The onset of spoilage in eggs held at 30 and 37 C coincided with the union of the yolk and the shell membranes; this occurred on the 5th to 7th day in eggs held at 37 C, but not until the 10th to 15th day in eggs held at 30 C.

ACKNOWLEDGMENT

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LITERATURE CITED

AYRES, J. C., AND B. TAYLOR. 1956. Effect of temperature on microbial proliferation in shell eggs. Appl. Microbiol. **4**:355–359.

BALLS, A. K., AND T. L. SWENSON. 1934. The antitrypsin of egg white. J. Biol. Chem. **106**:409–419.

BIGLAND, C. H., AND G. PAPAS. 1953. Experiment in egg penetration by salmonella. Can. J. Comp. Med. Vet. Sci. **17**:105–109.

BOARD, R. G. 1962. A study of bacterial infection of the hen's egg. Thesis, University of Edinburgh, Scotland.

BOARD, R. G. 1964. The growth of gram negative bacteria in the hen's egg. J. Appl. Bacteriol. **27**:350–364.

BOARD, R. G., J. C. AYRES, A. A. KRAFT, AND R. H. FORSYTHE. 1964. The microbiological contamination of egg shells and egg packing materials. Poultry Sci. **43**:584–595.

BROOKS, J. 1960. Mechanism of the multiplication of Pseudomonas in the hen's egg. J. Appl. Bacteriol. **23**:499–509.

BRANT, A. W., AND P. B. STARR. 1962. Some physical factors related to egg spoilage. Poultry Sci. **41**:1468–1473.

EAKIN, R. E., E. E. SNELL, AND R. J. WILLIAMS. 1940. A constituent of raw egg white capable of inactivating biotin *in vitro*. J. Biol. Chem. **136**: 801–802.

ELLIOTT, L. E., AND A. W. BRANT. 1957. Effect of saline and egg shell membrane on bacterial growth. Food Res. **22**:241–248.

FROMM, D., AND R. J. MONROE. 1960. Interior physical quality and bacterial contamination of market eggs as influenced by egg shell permeability. Food Technol. **14**:401–403.

FEENEY, R. E., AND D. A. NAGY. 1952. The antibacterial activity of the egg white protein conalbumin. J. Bacteriol. **64**:629–643.

GARIBALDI, J. A. 1960. Factors in egg white which control growth of bacteria. Food Res. **25**:337–344.

GARIBALDI, J. A., AND J. L. STOKES. 1958. Protective role of shell membranes in bacterial spoilage of eggs. Food Res. **23**:283–290.

GARIBALDI, J. A., AND H. G. BAYNE. 1960. The effect of iron on the *Pseudomonas* spoilage of experimentally infected shell eggs. Poultry Sci. **39**:1517–1520.

GARIBALDI, J. A., AND H. G. BAYNE. 1962*a*. The effect of iron on the *Pseudomonas* spoilage of farm washed eggs. Poultry Sci. **41**:850–853.

GARIBALDI, J. A., AND H. G. BAYNE. 1962*b*. Iron and the bacterial spoilage of shell eggs. J. Food Sci. **27**:57–59.

GILLESPIE, J. M., AND W. J. SCOTT. 1950. Studies in the preservation of shell eggs. IV. Experiments in the mode of infection by bacteria. Australian J. Appl. Sci. **1**:514–530.

HAINES, R. B. 1939. Microbiology in the preservation of the hen's egg. Rept. Food Invest. Board, No. 47. H.M.S.O., London.

HEALY, D. J., AND A. M. PETER. 1925. The hydrogen ion concentration and basicity of egg yolk and egg white. Am. J. Physiol. **74**:363–368.

LASCHTSCHENKO, P. 1909. Über die keimtotende und entwicklungschemmende Wirkung Hunereiweiss. Z. Hyg. Infektionskrankh. **64**:419–427.

LINEWEAVER, J., AND C. W. MURRAY. 1947. Identification of the trypsin inhibitor of egg white with ovomucoid. J. Biol. Chem. **71**:565–581.

MATSUSHIMA, K. 1958. An undescribed trypsin inhibitor in egg white. Science **127**:1178–1179.

MILES, A. A., AND E. T. HALNAN. 1937. A new species of microorganism (*Proteus melanovogenes*) causing black rot in eggs. J. Hyg. **37**:79–97.

OREL, V. 1959. The pseudomonas spoilage of eggs laid by individual hens. Poultry Sci. **38**:8–12.

RHODES, M. B., N. BENNETT, AND R. E. FEENEY. 1959. The flavoprotein-apoprotein system of egg white. J. Biol. Chem. **234**:2054–2060.

RHODES, M. B., N. BENNETT, AND R. E. FEENEY. 1960. The trypsin and chymotrypsin inhibitors from avian egg whites. J. Biol. Chem. **235**:1686–1693.

SALTON, M. R. J. 1957. The properties of lysoyme and its action on microorganisms. Bacteriol. Rev. **21**:82–100.

SCHADE, A. L., AND L. CAROLINE. 1944. Raw hen

egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. Science **100**:14–15.

SHARP, P. F., AND R. WHITAKER. 1927. The relation of hydrogen iron concentration of egg white to its germicidal action. J. Bacteriol. **14**:17–46.

STOKES, J. L., AND W. W. OSBORNE. 1956. Effect of the egg shell membrane on bacteria. Food Res. **21**:264–269.

STOKES, J. L., W. W. OSBORNE, AND H. G. BAYNE. 1956. Penetration and growth of *Salmonella* in shell eggs. Food Res. **21**:510–518.

STUART, L. S., AND E. H. MCNALLY. 1943. Bacteriological studies on the egg shell. U.S. Egg Poultry Mag. **49**:28–47.

WESLEY, R. L., AND W. I. STADELMAN. 1959. Measurements of interior egg quality. Poultry Sci. **38**:474–481.

WOLK, J., E. H. MCNALLY, AND N. H. SPICKNALL. 1950. The effect of temperature on the bacterial infection of shell eggs. Food Technol. **4**:316–318.

ZAGAEVSKY, J. S., AND P. O. LUTIKOVA. 1944. Sanitary measures in the egg breaking plant. U.S. Egg Poultry Mag. **50**:17–123.

Br. Poult. Sci., 13: 33-43. 1972 *Longman: printed in Great Britain*

THE BEHAVIOUR OF MIXED BACTERIAL INFECTIONS IN THE SHELL MEMBRANES OF THE HEN'S EGG

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SYNOPSIS

The selection of Gram-negative bacteria from a heterogenous flora on the shell membranes of the hen's egg occurred during two phases of the infection process. When the infection was confined to the shell membranes, the Gram-negative bacteria achieved dominance over the Gram-positive organisms. This dominance was accentuated when the albumen was invaded and it was noted, moreover, that a particular strain of Gram-negative bacteria predominated whereas several strains co-existed in the shell membranes.

INTRODUCTION

The shell of the hen's egg acquires, by contact, a heterogenous flora in which Gram-positive bacteria predominate (Haines, 1938; Board, Ayres, Kraft and Forsythe, 1964). The contents of rotten eggs harbour a mixed population of Gram-negative bacteria (Haines, 1938; Florian and Trussell, 1957; Board, 1965; Board and Board, 1968; Seviour, Sykes and Board, unpublished). There would appear to be no reason for ascribing the selection of the latter to agencies, such as washing, which can cause contamination of the membranes underlying the pores in the shell. When the pores are flooded, organisms are passively borne in the water which is sucked into the pore canals when a warm egg contracts in cold water or drawn in by capillarity when the egg and water are at the same temperature. The infection remains confined to the shell membranes for 10 or 20 d or more in eggs stored at room temperature (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Miller and Crawford, 1953; Stokes, Osborne and Bayne, 1956; Orel, 1959; Fromm and Monroe, 1960; Garibaldi and Bayne, 1960; Rizk, Ayres and Kraft, 1966; Büchli, 1967; Vadehra, Baker and Naylor, 1970). The initial contaminants of the albumen remain quiescent and the albumen harbours few bacteria until the yolk and shell membranes make contact (Board, 1964; Board and Ayres, 1965; Board, Hendon and Board, 1968). Large populations form quickly in the albumen in the 24 h following infection of the yolk's surface and, when the principal contaminants are proteolytic, lecithinolytic and/or pigmented, there are macroscopic changes in the yolk and white.

The present study was concerned with the behaviour of mixed bacterial populations placed on the shell membranes and it sought to identify the phase(s) of the infection process during which the selection of Gram-negative and, hence,

rot-producing bacteria occurred. When this study was begun, it was decided to use inocula prepared from the shells of dirty eggs instead of mixtures of laboratory cultures because it was considered that the latter approach would permit a poor simulation of the physiological state of the naturally occurring contaminants. The preparation of inocula from shell eggs did, however, mean that at the beginning of an experiment one had no idea of the numbers or types of organisms in an inoculum. It was for this reason that the experiments were done over a period of 3 years and the inocula were prepared from eggs coming from many sources. The results are discussed in this report.

MATERIALS AND METHODS

Preparation of inoculum

The shells of 3 dirty eggs and 100 ml of sterile distilled water were blended at low speed for 1 min in a domestic macerator ("Osterizer", John Oster, Milwaukee, USA). The fragmented shells and membranes were allowed to settle and the turbid supernatant used as the inoculum.

Eggs

These came from unmated flocks (housed in batteries) of commercial laying stock and they were 3 d-old at the time of inoculation. All eggs were candled and those of poor internal quality discarded. The shell above the air space was swabbed with ethanol and pierced with a rapidly rotating grinding-disc—the underlying membrane was not perforated. A sterile hypodermic syringe (B—D Plastipak, Becton Dickinson, Ireland) was used to inject the supernatant (0·1 ml) into the air space. The hole in the shell was closed with sterile paraffin wax and the eggs held at the temperatures noted in the text.

Sampling

At frequent intervals, 3 or 5 eggs were randomly selected, the shells broken and the contents collected in sterile Petri dishes. The exposed surface of the inner membrane of the air space was rinsed with sterile distilled water and removed. The membranes from the eggs were ground to a paste with a pestle in a mortar containing a few grains of sterile sand. The pestle and mortar were sterilised with formaldehyde and flushed with sterile distilled water. The paste was suspended in 9 ml of one-quarter-strength Ringer's solution or 0·1% (w/v) peptone water and serial decimal dilutions prepared in these diluents.

The albumen from all the eggs was transferred to a sterile Erlenmeyer flask containing a few glass beads. After shaking to mix, serial decimal dilutions were prepared in quarter-strength Ringer's solution.

Viable counts

The media were obtained from Oxoid Ltd and they were prepared according to the manufacturer's instruction. One millilitre of appropriate dilutions was used in the preparation of pour plates with nutrient agar (3 d incubation at 27 °C; the general count); nutrient agar containing 6% (w/v) NaCl (3 d at 27 °C; the micrococci count), and violet red bile agar (2 d at 37 °C; the coliform count).

RESULTS

Changes in microbial population size

Nutrient agar (incubation period 3 d at 27 °C) was used to monitor changes in the size of the microbial populations in the inner membrane of the air space of eggs which had been seeded with the flora present on the shells of dirty eggs. To retard colonisation of the albumen, the eggs were kept with their air spaces facing downwards. When dirty eggs from intensively housed hens provided the inoculum for eggs held in a domestic refrigerator (4 °C) there was a pronounced decline in the size of the populations in the inner membrane of the air cell (Figure 1). On one

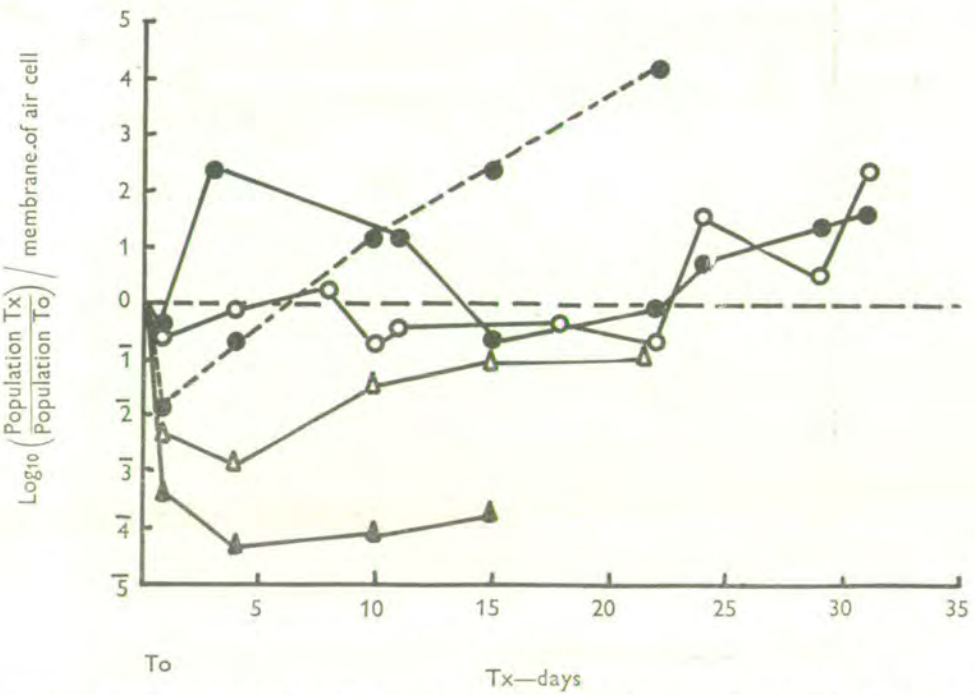

FIG. 1.—Relative changes in the size of bacterial populations in the inner membrane of the air space of the hen's egg. The inoculum was prepared from the shells of eggs laid by intensively housed hens. Each point represents the results obtained with a complete sample of 3 eggs. Eggs held at: ▲ = 4 °C, ● = 22 °C, ○ = 30 °C, △ = 37 °C, solid line = air cell lowermost, broken line = air cell uppermost.

occasion this continued until no viable organisms developed on nutrient agar to which had been added 1 ml of a decimal dilution of the homogenised membranes. In the majority of experiments, however, the decline ended by the 5th d and there was then a slow increase in the number of bacteria recovered from the inner membrane of the air space. A different trend was noted in refrigerated eggs which had been infected with the comminuted shells of eggs produced by free range hens (Figure 2). After a slight decrease in the size of the population, bacterial multiplication occurred during the 28 d of storage and the pattern given by the organisms in the cold-stored eggs was essentially the same as that of those in eggs stored at 27 °C. It was noteworthy that in this experiment, the albumen became heavily contaminated with pigment-producing pseudomonads. These observations indicate that, as

would be expected, the composition of the inoculum was greatly influenced by the circumstances which lead to the dirtying of the eggs. The shells of eggs of free-range hens harboured psychrophilic pseudomonads which quickly formed large populations in the cold-stored eggs whereas these organisms appeared to be absent from eggs produced by intensively housed hens.

A marked reduction in size was a feature of mixed populations added to the shell membranes of eggs held at 37 °C (Figure 1). Bacterial multiplication occurred

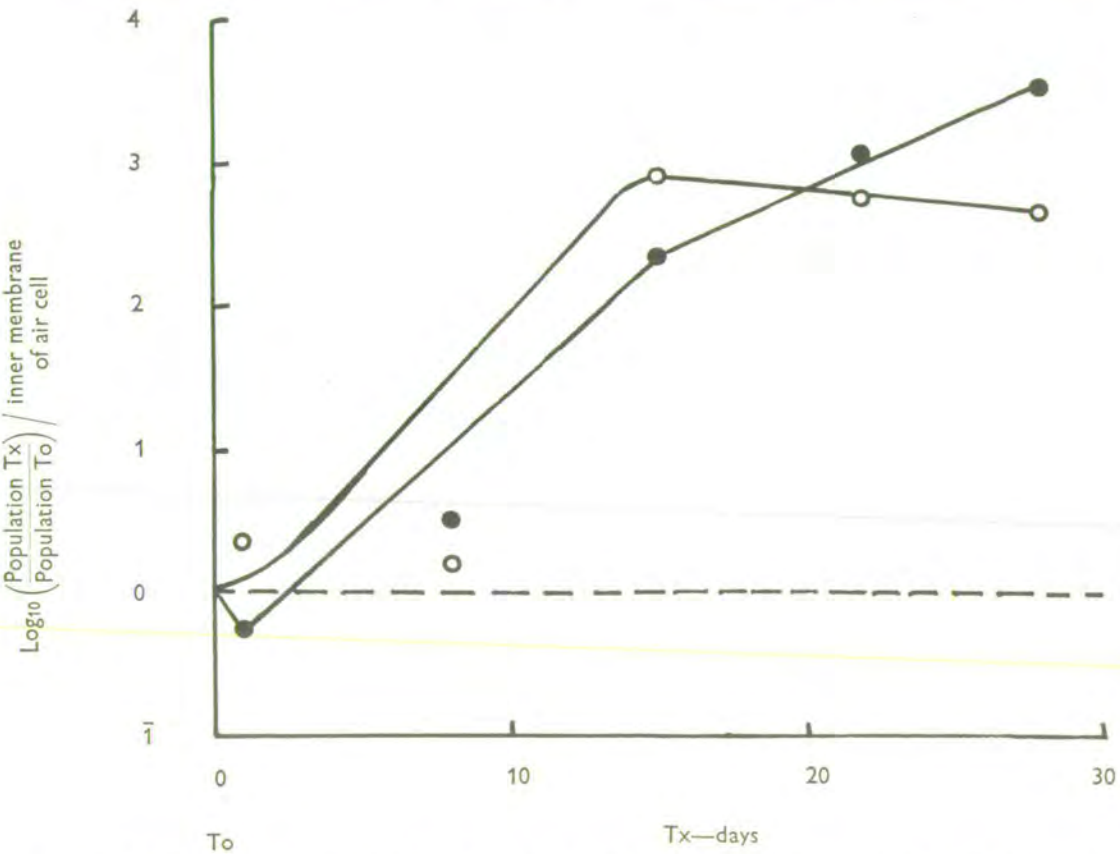

FIG. 2.—Relative changes in the size of the bacterial populations in the inner membrane of the air space of eggs. The inoculum was prepared from the shells of eggs laid by free range hens. Each point is the result obtained with a composite sample of 5 eggs. The eggs were held with their air spaces downwards. Eggs stored at: ● = 4 °C, ○ = 27 °C.

from the 5th d of storage onwards but the populations did not achieve a size equal to that of the inoculum. The extent of reduction in the size of populations seeded in eggs held at 22 °C or 30 °C (Figure 1) was less than that in eggs held at 4 °C or 37 °C. Moreover, bacterial multiplication resulted in the populations reverting to a size equal to or greater than that present in the inoculum.

Changes in the composition of the populations. To follow changes in the gross composition of the flora, 10 or 20 colonies were randomly selected from the nutrient agar at each sampling. Films were prepared and stained by Gram's method or, after

being replated to ensure purity, the isolates were characterised in detail sufficient to assign them to genera. There is a common trend in the results of the four experiments given in Figure 3. As would be expected (Haines, 1938; Board *et al.*, 1964), Gram-positive bacteria were plentiful in the inoculum prepared from the shells of dirty eggs. Their incidence of recovery decreased during storage until eventually Gram-negative bacteria alone were recovered. Pseudomonads became dominant

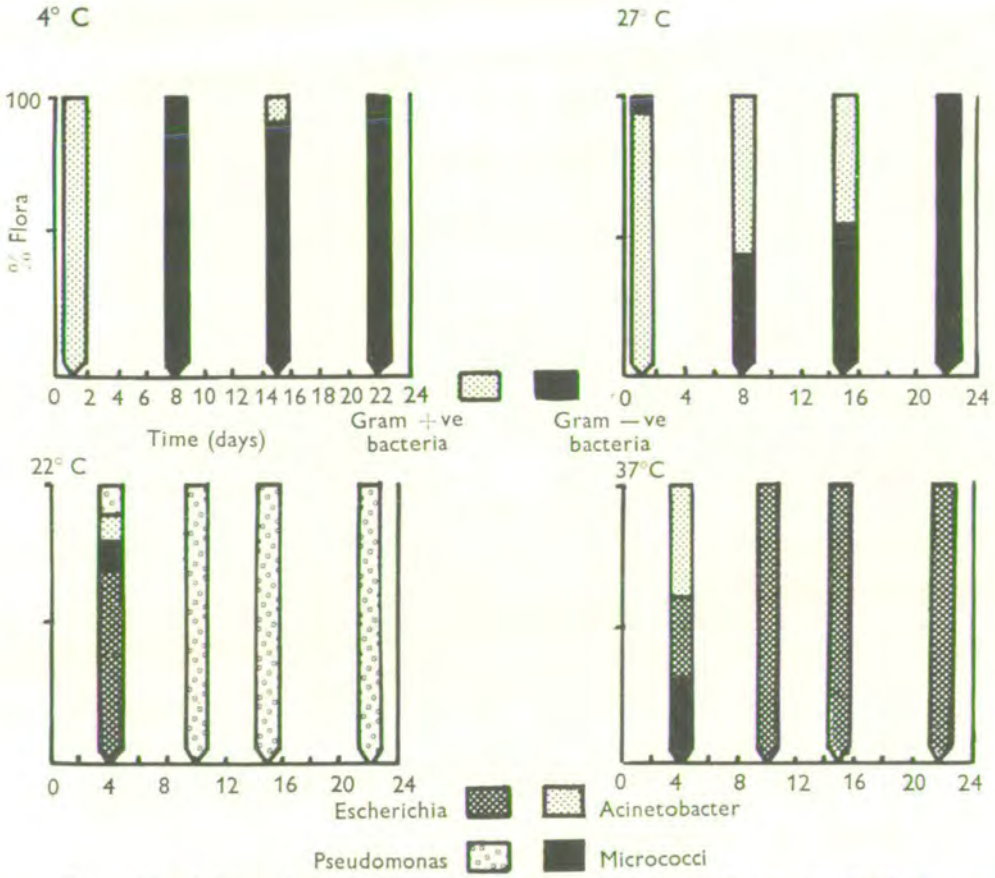

FIG. 3.—Compositional changes in the flora present in the inner membrane of the air space (held downwards) of eggs which had been inoculated with bacteria harvested from the shells of dirty eggs.

in eggs stored at room temperature or less whereas coliforms were predominant in the shell membranes of eggs stored at 37 °C.

Although this evidence indicates that Gram-negative bacteria became dominant within a few days following infection of the shell membrane, it does not indicate the fate of other components of the inoculum. That of the micrococci was followed on nutrient agar containing 6% (w/v) NaCl. In the experiments summarised in Figure 4, these organisms dominated the inoculum but their numbers decreased in the 24 h following the seeding of the air space of eggs stored at 22 °C or 30 °C. There was a progressive decrease in their numbers in eggs stored at 30 °C but it was interrupted on the second or third day of storage at 22 °C (Figure 5). At both temperatures,

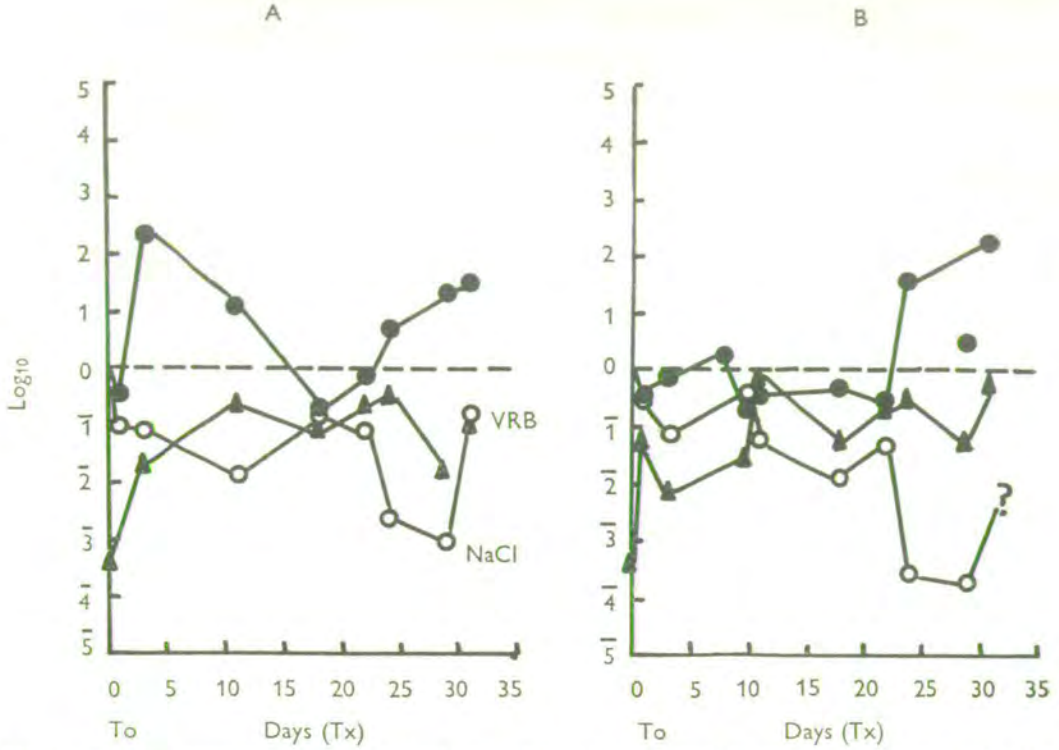

FIG. 4.—Relative contribution of micrococci and coliform bacteria to the populations present in the shell membranes of eggs held at 22 °C (A) or 30 °C (B). Each point represents the results obtained with a composite sample of the inner membrane of the air space of 3 eggs. Closed circles, relative change (population at Tx/population at To) of the general contaminants; open circles, number of micrococci/number of general contaminants per shell membrane, and closed triangle, number of coliform organisms/number of general contaminants per shell membrane.

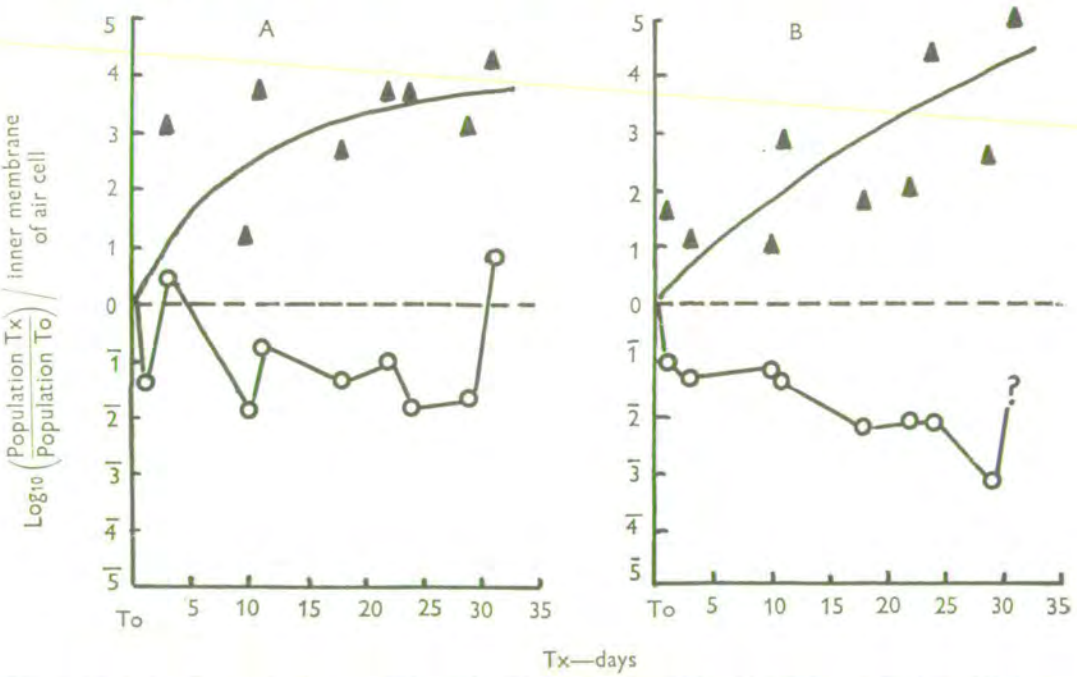

FIG. 5.—Relative changes in the populations of coliform organisms (closed triangles) and micrococci (open circles) in the inner membrane of the air space of eggs held at 22 °C (A) or 30 °C(B).

an increase in the number of micrococci occurred when eggs were stored for more than 30 d with their air spaces downwards. This increase occurred subsequent to the onset of the second phase of multiplication of the general contaminants. Likewise in eggs stored at 22 °C with their air spaces uppermost (Figure 6), the second phase

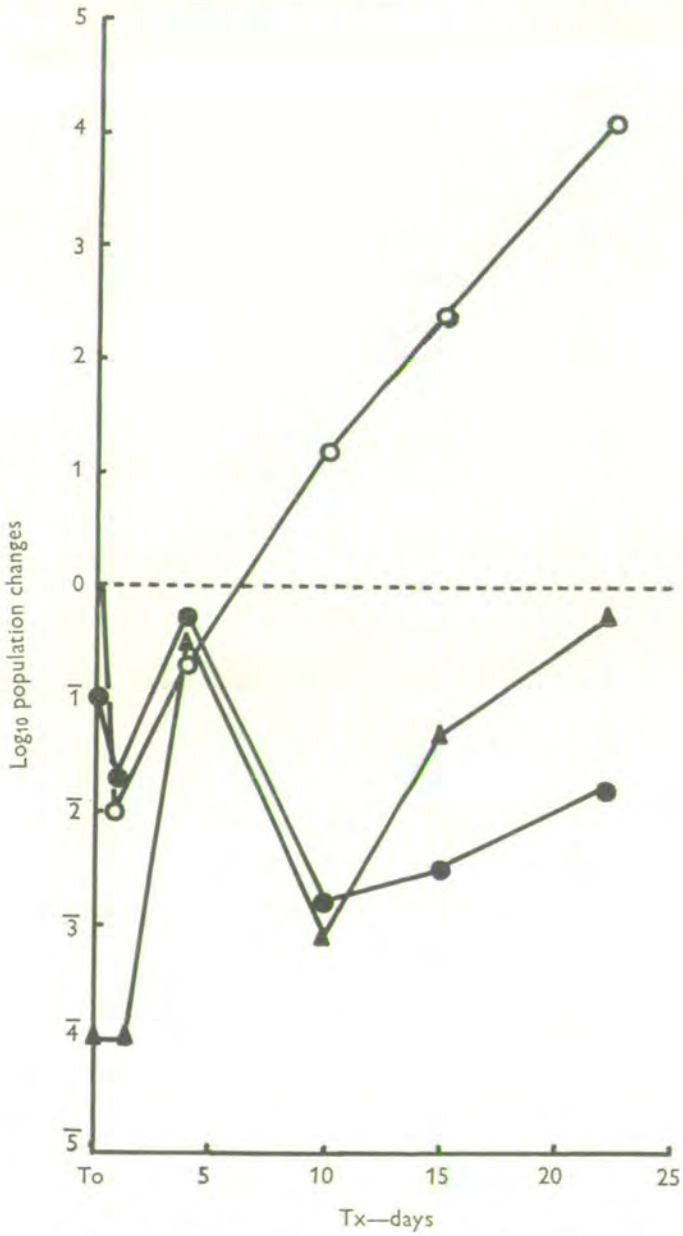

FIG. 6.—Relative contribution of micrococci and coliform organisms to the populations present in the shell membranes of eggs held with their air spaces uppermost at 22 °C. Open circles, relative change (population at Tx/population at To) of the general contaminants (Nutrient agar); closed circles, number of micrococci at Tx/number of general contaminants at Tx, and closed triangles, number of coliform organisms at Tx/number of general contaminants at Tx.

of multiplication of the micrococci did not occur until 2 to 3 d after the onset of rapid growth of the general contaminants which had been induced by the yolk making contact with the infected membrane Even then, the micrococci made only a paltry contribution to overall contamination of the inner membrane of the air cell. They

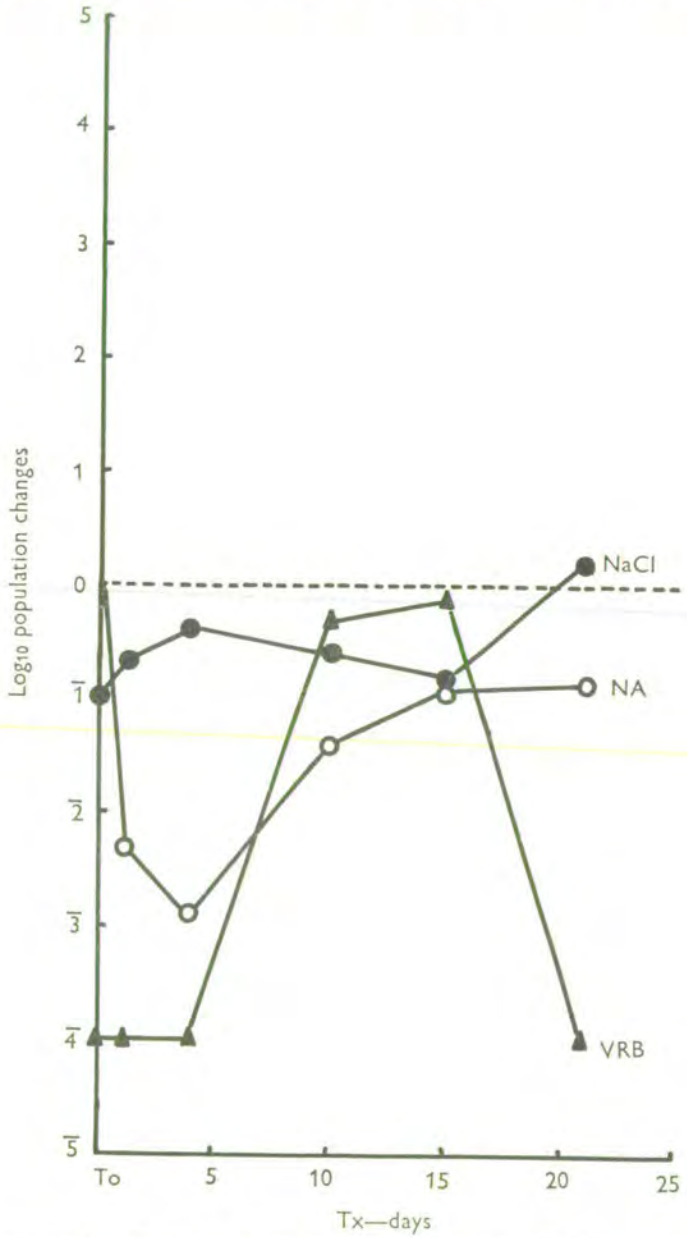

FIG. 7.—Relative contribution of micrococci and coliform organisms to the populations present in the shell membranes of eggs held with their air spaces downwards at 37 °C. Open circles, changes in the populations of the general contaminants (population at Tx/population at To); closed circles, number of micrococci at Tx/number of general contaminants at Tx, and closed triangles, number of coliform organisms at Tx/number of general contaminants at Tx.

did, however, become the predominant contaminants in eggs stored at 37 °C in a bacteriological incubator having no humidity control. In the experiment summarised in Figure 7, the number of micrococci in the inoculum was 1 log cycle less than the general count and it was noteable that their contribution to the flora remained at this level until the 21st d of storage at which time they were the principal contaminants. The eggs sampled at this time were desiccated and it was concluded that this had contributed to the selection of organisms having a relatively low a_w requirement.

In all the experiments, coliforms (recovered on violet red bile agar at 37 °C) made a negligible contribution to the inoculum. In contrast to the micrococci, however, their contribution to the infection of the inner membrane of the air space

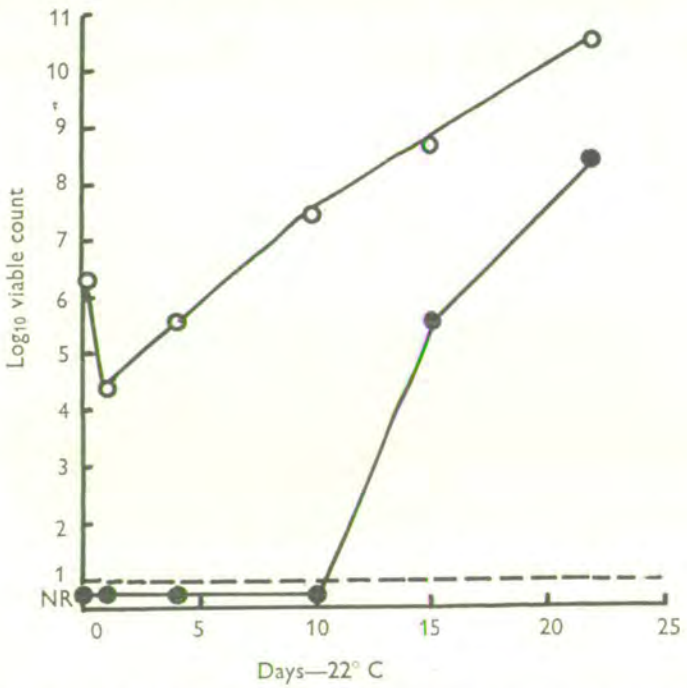

FIG. 8.—Changes in the size of the microbial populations in the inner membrane (open circles) and albumen (closed circles) of eggs held at 22 °C with their air spaces uppermost. NR, viable organisms not recovered.

increased throughout the storage of eggs at 22 °C, 30 °C or 37 °C (Figures, 4, 6 and 7). This was reflected in the progressive increase in the number of coliforms when the numbers recovered at each sampling were compared with those in the inoculum (Figure 5). Nevertheless, the increase in the numbers of coliforms did not lead to their dominance in eggs held at 22 °C or 30 °C. Moreover, when the union of the yolk and infected membranes in eggs stored at 22 °C induced a rapid increase in the numbers of general contaminants (Figure 6), the rapid growth of coliforms was not noted until about 5 d later. The coliforms did achieve dominance in the flora present in the shell membranes of eggs stored for 10 to 15 d at 37 °C (Figure 7) but, with the desiccation noted previously, there was a pronounced decrease in their numbers and they were replaced by micrococci.

In the work discussed above, particular attention was given to the behaviour of

mixed populations of bacteria in eggs in which the yolk moved away from the site of infection. In one experiment the eggs were stored with their air spaces uppermost. The changes (Figure 8) in the size of the populations in the air cell membrane and albumen were essentially the same as those discussed elsewhere (Board, 1964). It was noted that pseudomonads became the predominant organism in the shell membrane and, judging from the form of the colony, several strains were harboured by the membranes. Only one colony form was recovered from the albumen following the union of the yolk and the infected shell membrane.

DISCUSSION

This investigation has shown that Gram-negative bacteria were selected during two phases in the process of infection of the hen's egg. The initial selection occurred when the infection was confined to the shell membranes. It was characterised by changes in the relative proportions of the components of the microbial population rather than by the elimination of micro-organisms other than Gram-negative bacteria. Moreover, temperature was an important selective factor. In eggs stored at 37 °C, the coliform organisms achieved dominance whereas at 30 °C or less it was the pseudomonads. The change in the relative proportions of organisms was not, however, permanent. Thus with eggs stored at 37 °C, the coliform organisms achieved dominance within a few days of the shell membranes being contaminated but they had been replaced by micrococci by the 21st d of incubation. It was surmised that the latter has been selected when the a_w fell due to evaporation. The second phase of selection occurred following the union of the yolk and shell membranes and it was noted that only one of the many strains of the Gram-negative bacteria present in the shell membranes contributed to the large populations which formed in the albumen.

When the shell membranes are suspended in a solution of mineral salts, they support the growth of the commonly occurring rot-producing bacteria (Board, 1965). The membranes *in situ* allow only limited microbial growth (Board, 1964) and this has been attributed (Board, 1969) to the environment within the shell membranes being adversely influenced by the antimicrobial substances in the albumen. Conalbumin (ovotransferrin) appears to be the principal bacteriostatic agent, its affect on Gram-negative bacteria being less than that on Gram-positive organisms (Feeney and Nagy, 1952). It is noteworthy that in this study the number of Gram-negative bacteria in the shell membrane increased whereas there was a decrease in the numbers of Gram-positive bacteria. Such changes in the relative proportions of Gram-negative to Gram-positive bacteria are common during the spoilage of proteinaceous foods such as meat (Ayres, 1960), fish (Shewan, 1961), etc., and it is generally accepted that the faster growth rate of the Gram-negative bacteria results in their dominance over Gram-positive bacteria. Such an interpretation would seem to be applicable to our observations, particularly as the competition is between Gram-negative rods and micrococci, a group of organisms for which the absence of a well-defined niche caused Niel (1955) to wonder as to their evolution and continued existence in such large numbers.

ACKNOWLEDGEMENT

The authors wish to express their thanks to the British Egg Marketing Board for financial support.

REFERENCES

AYRES, J. C. (1960). Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. *Fd Res.*, **25**: 1-18.

BIGLAND, C. H. AND PAPAS, G. (1953). Experiment in egg penetration by *Salmonella*. *Can. J. comp. Med.*, **17**: 105-109.

BOARD, P. A. AND BOARD, R. G. (1968). A diagnostic key for identifying organisms recovered from rotten eggs. *Br. Poult. Sci.*, **9**: 111-120.

BOARD, P. A., HENDON, L. P. AND BOARD, R. G. (1968). The influence of iron on the course of bacterial infection of the hen's egg. *Br. Poult. Sci.*, **9**: 211-215.

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.*, **27**: 350-364.

BOARD, R. G. (1965). Bacterial growth on and penetration of the shell membranes of the hen's egg. *J. appl. Bact.*, **28**: 197-205.

BOARD, R. G. (1969). The microbiology of the hen's egg. *Adv. appl. Microbiol.*, **11**: 245-281.

BOARD, R. G. AND AYRES, J. C. (1965). Influence of temperature on bacterial infection of the hen's egg. *Appl. Microbiol.*, **13**: 358-364.

BOARD, R. G., AYRES, J. C., KRAFT, A. A. AND FORSYTHE, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.*, **43**: 584-595.

BÜCHLI, K. (1967). De invloed van het wassen ip de microbiologische houd baar heid van het ei. Instituut voor Pluimveeteelt " Het Spelderholt "—Beekbergen Mededeling No. **145**.

FEENEY, R. E. AND NAGY, D. A. (1952). The antibacterial activity of the egg white protein conalbumin. *J. Bact.*, **64**: 629-643.

FLORIAN, M. L. E. AND TRUSSELL, P. C. (1957). Bacterial spoilage of eggs. IV. Identification of spoilage organisms. *Fd Technol., Champaign*, **11**: 56-60.

FROMM, D. AND MONROE, R. J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg-shell permeability. *Fd Technol., Champaign*, **14**: 401-403.

GARIBALDI, J. A. AND BAYNE, H. G. (1960). The effect of iron on pseudomonas spoilage of experimentally infected eggs. *Poult. Sci.*, **39**: 1517-1520.

GILLESPIE, J. H. AND SCOTT, W. J. (1950). Studies in the preservation of shell eggs. IV. Experiment on the mode of infection by bacteria. *Aust. J. appl. Sci.*, **1**: 514-530.

HAINES, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of a new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.*, **38**: 338-355.

MILLER, W. A. AND CRAWFORD, L. B. (1953). Some factors influencing bacterial population in eggs. *Poult. Sci.*, **32**: 303-309.

NIEL, C. B. VAN (1955). Natural selection in the microbial world. *J. gen. Microbiol.*, **13**: 201-217.

OREL, V. (1959). The pseudomonas spoilage of eggs laid by individual hens. *Poult. Sci.*, **38**: 3-12.

RIZK, S. S., AYRES, J. C. AND KRAFT, A. A. (1966). Effect of holding conditions on the development of salmonellae in artificially inoculated hen's eggs. *Poult. Sci.*, **45**: 825-829.

SHEWAN, J. H. (1961). The microbiology of sea-water fish. In: *Fish as Food*, Vol. 1, pp. 487-560. London, Academic Press.

STOKES, J. L., OSBORNE, W. W. AND BAYNE, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Fd Res.*, **21**: 510-518.

VADEHRA, D. V., BAKER, R. C. AND NAYLOR, H. B. (1970). Infection routes of bacteria into chicken eggs. *J. Fd Sci.*, **35**: 61-62.

ZAGAEVSKY, J. S. AND LUTIKOVA, P. O. (1944). Sanitary measures in the egg breaking plant. *U.S. Egg Poult. Mag.*, **50**: 17-20, 43-46, 75-77, 89-90, 121-124.

RESEARCH NOTE

THE INFLUENCE OF IRON ON THE COURSE OF BACTERIAL INFECTION OF THE HEN'S EGG

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RESEARCH NOTE

THE INFLUENCE OF IRON ON THE COURSE OF BACTERIAL INFECTION OF THE HEN'S EGG

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NATURAL or artificial contamination of water with iron salts is known to increase the rate and incidence of rotting during the storage of washed eggs (Garibaldi and Bayne, 1960). In view of the commercial importance of this phenomenon, it is surprising that the underlying mechanism(s) have not been elucidated. The present communication presents data that allow a rational interpretation of this facet of egg microbiology.

EXPERIMENTAL

The methods used in this study have been described elsewhere (Board, 1964). Briefly, eggs obtained during one day from a mated flock were candled and those of poor internal quality discarded. The air spaces were inoculated with organisms that, after two washings in 0·067 M Sørensen's phosphate buffer, had been suspended in distilled water or a solution of $FeSO_4 \cdot 7H_2O$ (final concentration in air space, 5 μg.). The eggs, with their air spaces lowermost, were held at temperatures noted in the text. The course of infection was followed by the enumeration of viable organisms in the inner membrane of the air space and in the albumen.

The organisms were derived from the Department's collection of stock cultures (*Serratia marsescens*, *Salmonella waycross*) or from rotten eggs (*Pseudomonas aeruginosa*, 2 strains). They were maintained on slopes of nutrient agar (Oxoid, London) at 4° C. For experimental purposes an organism was grown in nutrient broth (Oxoid, London) at 27° C. for 18 hr.

RESULTS

The influence of iron on the behaviour of pseudomonas in eggs held at summer shade temperature (*c.* 20° C.) is exemplified by the data given in Figure 1 and Table 1. In eggs containing no additional iron, the populations in the inner membrane of the air cell (Figure 1) declined in the 24 hr following inoculation but increased in the 24-48 hr period. Thereafter there was no appreciable change. Significant contamination of the albumen was noted only on the 4th day of incubation. These results are in agreement with those discussed elsewhere (Brooks, 1960; Board, 1964). When the inoculum was prepared in a solution of $FeSO_4$, the lag phase of growth was followed by a 48 hr period during which there was extensive multiplication of the organisms in the inner membrane of the air cell. The latter

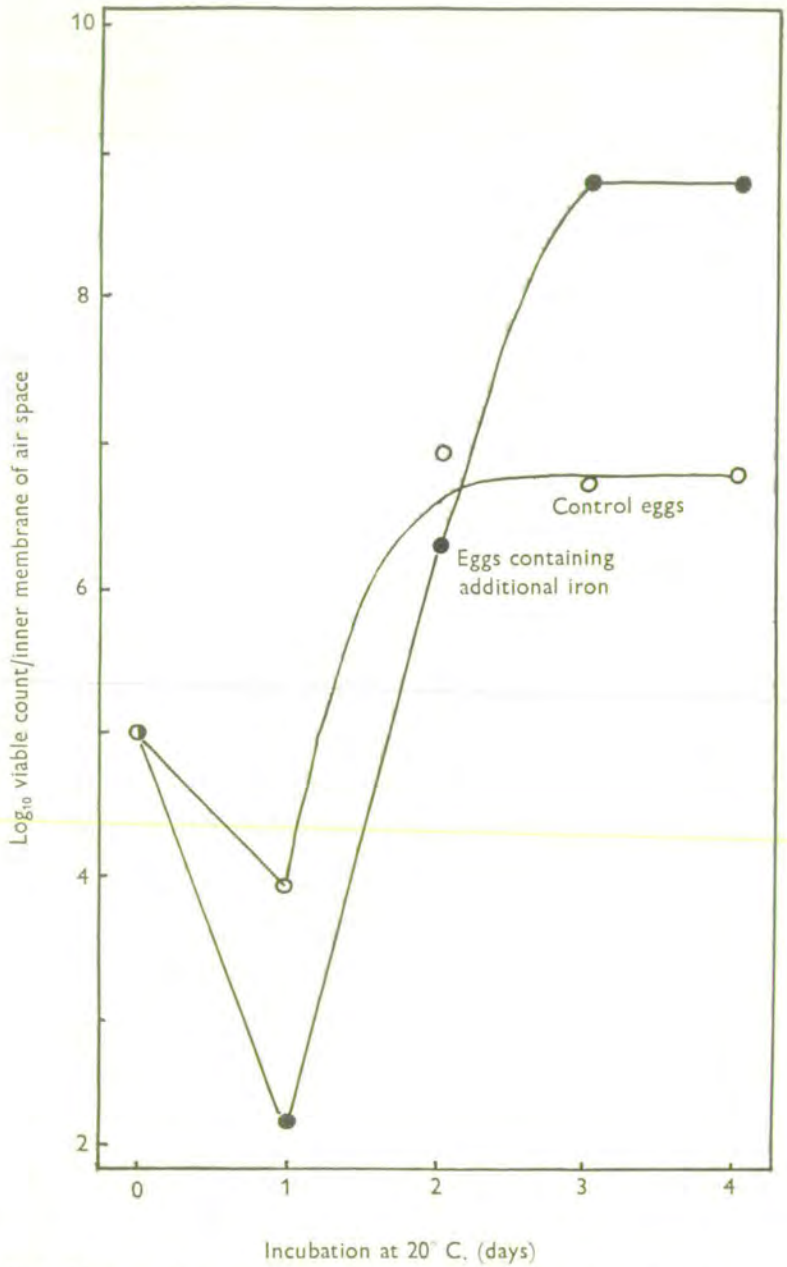

FIG. 1.—The effect of iron on the multiplication of *Pseudomonas aeruginosa* in the inner membrane of the air cell. The inner membrane of the air cell was contaminated with 5 μg. of iron, in the form of $FeSO_4.7H_2O$. Each point represents the average obtained from 3 eggs.

harboured more than 100 million viable organisms as compared with the 1 million in the membranes of control eggs. Moreover, there was a progressive increase in the level of contamination of the albumen (Table 1). By the 4th day of incubation the white contained more than 1 million viable organisms/ml. and it was stained throughout with the fluorescent pigments produced by *Ps. aeruginosa*. Results similar to these were obtained when eggs infected with *S. waycross* were held at 20° C.

TABLE 1

Influence of iron on contamination of albumen of hen's egg

Incubation at 20° C. (days)	Number of viable organisms in albumen of eggs infected in the air cell with *Pseudomonas aeruginosa* suspended in	
	distilled water	solution of $FeSO_4$*
0	NT	NT
1	+	+
2	+	$4{\cdot}2 \times 10^2$**
3	—	$6{\cdot}6 \times 10^3$
4	$4{\cdot}0 \times 10^2$	$4{\cdot}0 \times 10^6$***

NT, not tested; +, viable organisms recovered but counts not obtained because the viscosity of the albumen prevented a thorough mixing of the sample and nutrient agar; —, viable organisms not recovered; *, final concentration in air cell, 5 μg.; **, average obtained from 3 eggs; ***, albumen stained with fluorescent pigment.

In an attempt to give more emphasis to the promotion of microbial growth by iron, *Ser. marsescens* was chosen because of the observation (Board and Ayres, 1965) that this organism remains quiescent when eggs, containing no additional iron, are held at 10° C. From the results given in Figure 2 it will be seen that there was a slow but progressive increase in the populations in the inner shell membrane of eggs to which $FeSO_4$ had been added whereas there were no obvious trends in the populations in control eggs. With the latter, viable organisms were not recovered from the albumen until the 31st day following inoculation whereas heavy infection of the albumen of eggs containing additional iron was noted on the 10th day and it continued to increase until there was 1 million viable organisms/ml. on the 31st day of incubation. Infections of this magnitude were not associated with detectable changes in the H-ion or glucose content of the albumen until the 51st day following inoculation. Then the albumen had a *p*H of 8·8 and a glucose content of 1·05 mg/ml. as compared with 3·0 mg./ml. and *p*H 9·4 in the control eggs.

It has been reported (Board, 1964) that the shell membranes are stained by the addition of $FeSO_4$. This was seen in the present study and it was noted that the stain did not diminish even in eggs held at 10° C. for 8 weeks. Direct evidence of the localisation of iron in the shell membranes was presented by Garibaldi and Bayne (1962*a*, *b*). When eggs were soaked in water containing 10 p.p.m. iron, these workers did not detect a significant increase in the concentration of this element in the albumen.

The present study has shown that the addition of iron to an infected shell membrane promotes extensive bacterial multiplication and a heavy infection of the

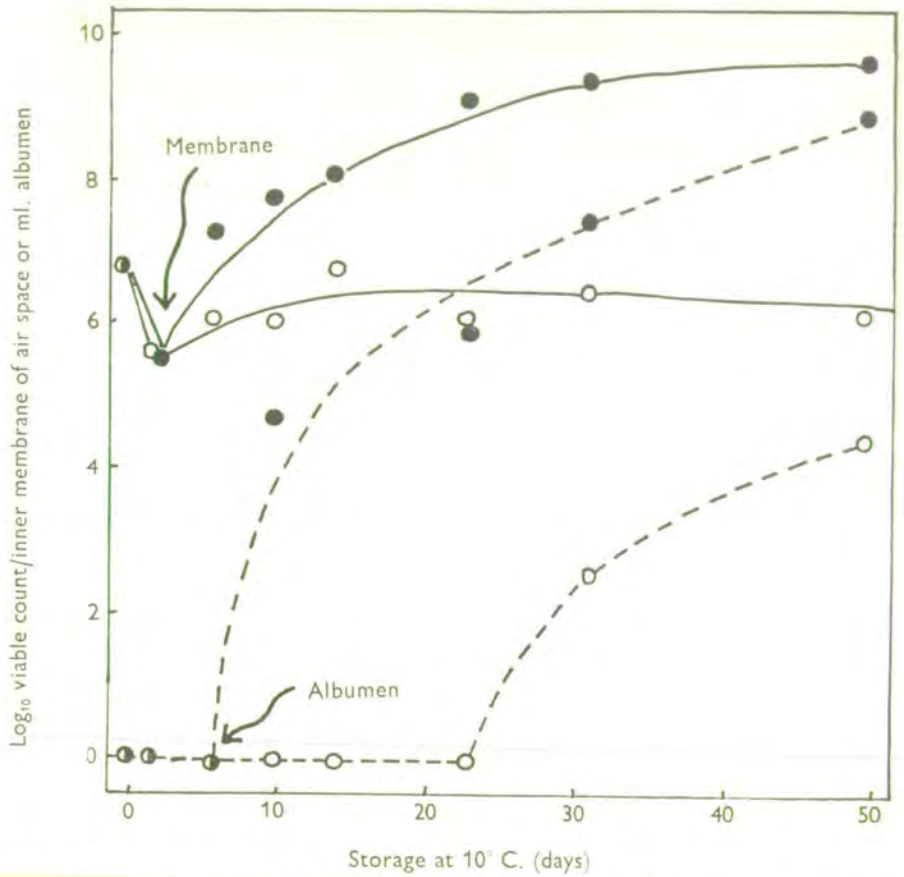

FIG. 2.—The influence of iron on the growth of *Serratia marsescens* in the hen's egg during incubation at 10° C. ●, Eggs in which the air cell membrane was contaminated with 5 μg. of iron; ○, Control eggs. Each point is the average obtained from 4 eggs.

albumen. Our failure to detect changes in the H-ion or glucose content of heavily contaminated albumen indicates that the initial contaminants of the white do not grow. The actual behaviour of these organisms will not be known until a technique has been devised whereby they can be studied *in ova*. At the moment it is considered that the organisms in the albumen remain quiescent until they collide with the yolk. They then multiply and, in the case of chromogenic or proteolytic contaminants, the first symptoms of rotting are manifested. In practice, therefore, the presence of iron in the water used for washing the egg leads to extensive multiplication of the organisms which penetrate the shell. The albumen becomes heavily infected thereby increasing the opportunity of collision of organism and the yolk.

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REFERENCES

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.*, **27**: 350-364.

BOARD, R. G. AND AYRES, J. C. (1965). The influence of temperature on bacterial infection of the hen's egg. *Appl. Microbiol.*, **13**: 358-364.

BROOKS, J. (1960). Mechanisms of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.*, **23**: 499-509.

GARIBALDI, J. A. AND BAYNE, H. G. (1960). The effect of iron on *Pseudomonas* spoilage of experimentally infected shell eggs. *Poult. Sci.*, **39**: 1517-1520.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*a*). Iron and the bacterial spoilage of shell eggs. *J. Fd Sci.*, **27**: 57-59.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*b*). The effect of iron on the *Pseudomonas* spoilage of farm washed eggs. *Poult. Sci.*, **41**: 850-853.

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A NOTE ON THE STRUCTURE AND IRON-BINDING PROPERTIES OF EGG-SHELL MEMBRANES

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1. When a solution of ferric ammonium sulphate was added to shell membranes of the domestic fowl, iron infiltrated the mantle (cortex) surrounding the cores of the individual fibres of the membranes.

2. Contraction of warm eggs in ice-cold colloidal iron caused flooding of pore canals and contamination of the underlying shell membranes with this element.

3. Appreciable contamination of the inner shell membrane with iron persisted for 25 d in infertile eggs stored at 37·5 °C.

INTRODUCTION

The egg-shell membranes of the domestic fowl consist of three distinct layers. The major ones, the inner and outer membranes, are formed from a network of randomly orientated fibres having their long axes parallel to the surface of the egg shell (Bellairs and Boyde, 1969). The diameter of the fibres range from 0·4 to 3·6 μm, the smaller ones being more numerous in the inner membrane. A homogenous third layer of electron dense material, the limiting membrane of Bellairs and Boyde (1969), lines the inner surface of the inner shell membrane.

Although the general morphology of the egg-shell membranes and their possible role in the transfer of Ca^{2+} from the shell of the embryo via the chorioallantois have been studied extensively (Masshoff and Stolpmann, 1961; Simkiss, 1968; Tung and Richards, 1972; Narbaitz and Tellier, 1974), little is known about their chemical composition particularly with respect to fine structure. The fibres of the inner and outer shell membranes have a protein core (the medulla) surrounded by a mucopolysaccharide mantle or cortex. Early investigators (Moran and Hale, 1936) concluded that the membranes contained a fibrous protein, probably keratin. The demonstration that the shell membranes are rich in cysteine (Baker and Balch, 1962) would support such a view. Recently, however, histochemical and ultrastructural analyses (Hoffer, 1971; Wedrel *et al.*, 1974) have not identified keratin, and the occurrence of the cross-linking amino acids, desmosine and isodesmosine, in membrane hydrolysates, suggest that elastin or an elastin-like protein is present (Leach and Rucker, 1978; Starcher and King, 1980). The demonstration that the

shell-membrane proteins are resistant to elastase and have an amino acid composition different from that of elastin and other known fibrous proteins (Leach *et al.*, 1981) indicates that the shell membranes of the hen's egg are formed of an as yet uncharacterised fibrous protein. The mantle on the fibres has not been characterised in detail; it probably contains glucose (Baker and Balch, 1962), galactose, mannose and xylulose (Wedral *et al.*, 1974) as well as glucosamine, galactosamine and sialic acid (Cooke and Balch, 1970).

Not only are the shell membranes involved in the diffusion of respiratory gases to and from the chorioallantois (Tullett and Board, 1976; Kayar *et al.*, 1981) and the movement of Ca^{2+} (Coleman and Terepka, 1972) but they play a physical role in protecting the albumen from microbial infection (Bean and MacLaury, 1959; Williams and Whittemore, 1967). Moreover bacteria placed on the inner shell membrane of the air space *in ovo* multiply to a limited extent (Brooks, 1960; Board and Ayres, 1965) unless supplied with Fe^{3+} (Board *et al.*, 1968) or suspended in soil or faecal extracts (Board, 1964). The last mentioned workers noted that the stain following the addition of iron to the shell membranes persisted for several days with incubation at 27 °C. The present study was undertaken with the objectives of identifying the sites(s) in the shell membranes where iron is harboured, and assessing the persistence of iron contamination of the shell membranes.

MATERIALS AND METHODS

Eggs

Good quality eggs and eggs with defective shells and cuticles were obtained from a commercial flock of laying hens, housed in batteries and fed on a proprietary diet. The eggs were used immediately or kept in an incubator (Brinsea Products Ltd, West Brinsea Farm, Congresbury, Avon), maintained at 37·5 °C and 0·60 relative humidity. The eggs were turned automatically every hour.

Glassware

All glassware used in the experiments involving iron was freed of contaminating iron by the following procedure: 1, an overnight soak in 0·1 M HCl; 2, a rinse with deionised, glass-distilled water; 3, a wash with 8-hydroxyquinoline (BDH Ltd) (5 g/l) in chloroform to chelate any iron present (Waring and Werkman, 1942); 4, a rinse in chloroform to remove any traces of chelate; 5, a dry in a hot-air oven to remove chloroform; 6, a double rinse with deionised, glass-distilled water and 7, a dry in a hot-air oven.

Iron solutions

A solution (6 mg/ml) of filter-sterilised (0·45 μm, Oxoid Ltd) $Fe(NH_4)_2(SO_4)_2.6H_2O$ was used to inoculate (0·1 ml) the inner shell membrane of the eggs. The needle of a hypodermic syringe was pushed through the outer shell membrane via a small hole drilled in the shell at the air space. The whole operation was done aseptically and the hole in the shell sealed with hot paraffin wax.

A colloidal iron suspension was prepared according to the method of Rinehart and Abul-Haj (1951). Fifty grams $FeCl_3.6H_2O$ was dissolved in 80 ml glass-distilled water and 40 ml glycerol added slowly. Twenty-two ml ammonia solution (280 ml/l) was carefully and slowly mixed into the iron suspension which was stirred continuously to prevent the formation of excessive precipitate. The whole suspension was dialysed against two changes of distilled water every day for three days—it can be kept for several months at 4 °C. Warm eggs (37·5 °C) placed in this cold iron suspension drew iron through the pores in the shell onto the shell membranes due to the pressure differential generated by the contraction of the egg contents.

Iron determination

Shell membranes containing bound iron were washed six times with deionised water to remove any adhering albumen and solubilised by boiling in ammonium persulphate (20 g/l). Albumen containing iron was mixed with an equal volume of ascorbate (BDH Ltd) (0·2 g/l) in 0·2 M HCl and allowed to stand for 10 min. The protein was precipitated using trichloroacetic acid (110 g/l) and removed by centrifugation (5 000 *g*).

To 0·5 ml of solubilised membrane or egg albumen supernatant was added 0·4 ml ammonium acetate (100 g/l) followed by 0·1 ml of iron reagent containing 3 mg/ml of ferrozine (Sigma Ltd) and neocuproine (Sigman Ltd). The absorbance of the magenta-coloured complex was measured at 562 nm against water. Iron concentrations were calculated from standards containing known amounts of iron processed in the same way as the test samples.

Electron microscopy

Samples of shell membranes for scanning electron microscopy were fixed in gluteraldehyde (20 g/l) in 0·05 M cacodylate buffer, pH 7·0, for 60 min at room temperature followed by dehydration in a series (50 to 100%) of acetone-water mixtures. The samples were then dried by the critical point method using a Polaron 5000 critical drying point apparatus and mounted on aluminium stubs using DAG 915 (Acheson Colloids Co., Prince Rock, Plymouth). The specimens were coated under vacuum with a thin layer of gold/palladium alloy and examined using a Joel 35C (Joel (UK) Ltd, Colindale, London) Scanning electron Microscope C at an accelerating voltage of 25 kV.

Samples of shell membranes for transmission electron microscopy were fixed and stained in gluteraldehyde (20 g/l) containing ruthenium red (1 g/l). After dehydration as described above the membranes were embedded in Taab EM resin (Taab Laboratories Equipment Ltd, Reading, Berkshire) and ultra-thin sections, cut with a glass knife, collected onto uncoated copper grids. Sections were stained with a saturated solution of uranyl acetate in ethanol (700 ml/l) and lead citrate for 15 min each and examined using the Joel 100C Transmission electron Microscope (Joel (UK) Ltd, Colindale, London) at an accelerating voltage of 20 kV. Some sections were stained with the colloidal iron suspension for 4 h before examination with the electron microscope.

OBSERVATIONS AND DISCUSSION

The cores and mantles of the fibres of the shell membranes included in this study are obvious features in Plate-Fig. 1A and B. When colloidal iron was used to stain membranes, the mantles were impregnated with this element to such an extent that their resistance to electron transmission was markedly reduced (Plate-Fig. 1C). It is evident from this illustration that the iron had not penetrated all the mantles to the same extent. Nevertheless, as all the mantles contained iron at their periphery, it would seem reasonable to conclude that this element would have been available to bacterial contaminants had they been present between or on the fibres. Indeed the illustration in Plate-Fig. 1C provides evidence of adhesion of bacteria to the mantle on the fibres.

Iron contamination of the shell membranes in commerce could result from washing eggs without adequate control of the temperature of the washing and rinsing water (Garibaldi and Bayne, 1962*a*,*b*). When warm (37·5 °C) eggs were submerged in ice-cold colloidal iron, the element was deposited on the membranes underlying pores which had been flooded (Plate-Fig. 2). This illustration shows moreover that both the incidence of pores flooded and the extent of Fe^{3+} contamination of the shell membranes were influenced by shell quality. Extensive contamination of the shell membranes with Fe^{3+} occurred when cracked eggs were submerged in colloidal iron (Plate-Fig. 2D). As it has been demonstrated (Board and Halls, 1973) that a stain, edicol pea green, particles of carbon black and cells of *Serratia marsescens* were invariably present in the membranes underlying pores that had been flooded by warm eggs in this mixture, it can be concluded that during the washing of eggs there may be an opportunity for iron to be deposited along with micro-organisms on the shell membranes.

Immersion of warm (37·5 °C) eggs in ice-cold extracts of soil or faecal material stained the shell membranes underlying some pores in the egg shell. In this case, however, the addition of potassium ferrocyanide (10 g/l) to the membranes did not demonstrate iron, probably because the reagent was not sufficiently sensitive.

Garibaldi and Bayne (1962*b*) were probably the first to note that both the incidence and rate of rotting of eggs washed under farm conditions were increased when the wash water contained 4·8 mg iron/l. Judging from the results of Board *et al.* (1968), iron contamination of the shell membranes enhanced the growth of bacteria trapped in the membranes and resulted in heavy contamination of the underlying albumen with organisms that probably remained quiescent because of iron being chelated by ovotransferrin. Board *et al.* (1968) first noted how the staining of shell membranes with iron persisted in eggs held at 10 °C even after eight weeks. In this study it was possible to calculate just how much iron remained bound to the shell membranes with the use of a sensitive reagent. The results presented in Fig. 1 show that the content of iron in the inner membrane of the air space declined from approximately 500 to 100 μg/ml of hydrolysate within 10 d of incubation at 37·5 °C; thereafter the degree of contamination remained at about 100 μg/ml. It was noted that some of the iron added to the air space was retained by the outer shell membrane as it was pulled away from the inner one during the enlargement of the air space. Some of the iron moved from the shell membranes into the albumen (Fig. 2) but the peak concentration in the latter (*i.e.* 150 μg/ml on day 17) would not have been sufficient to saturate the ovotransferrin.

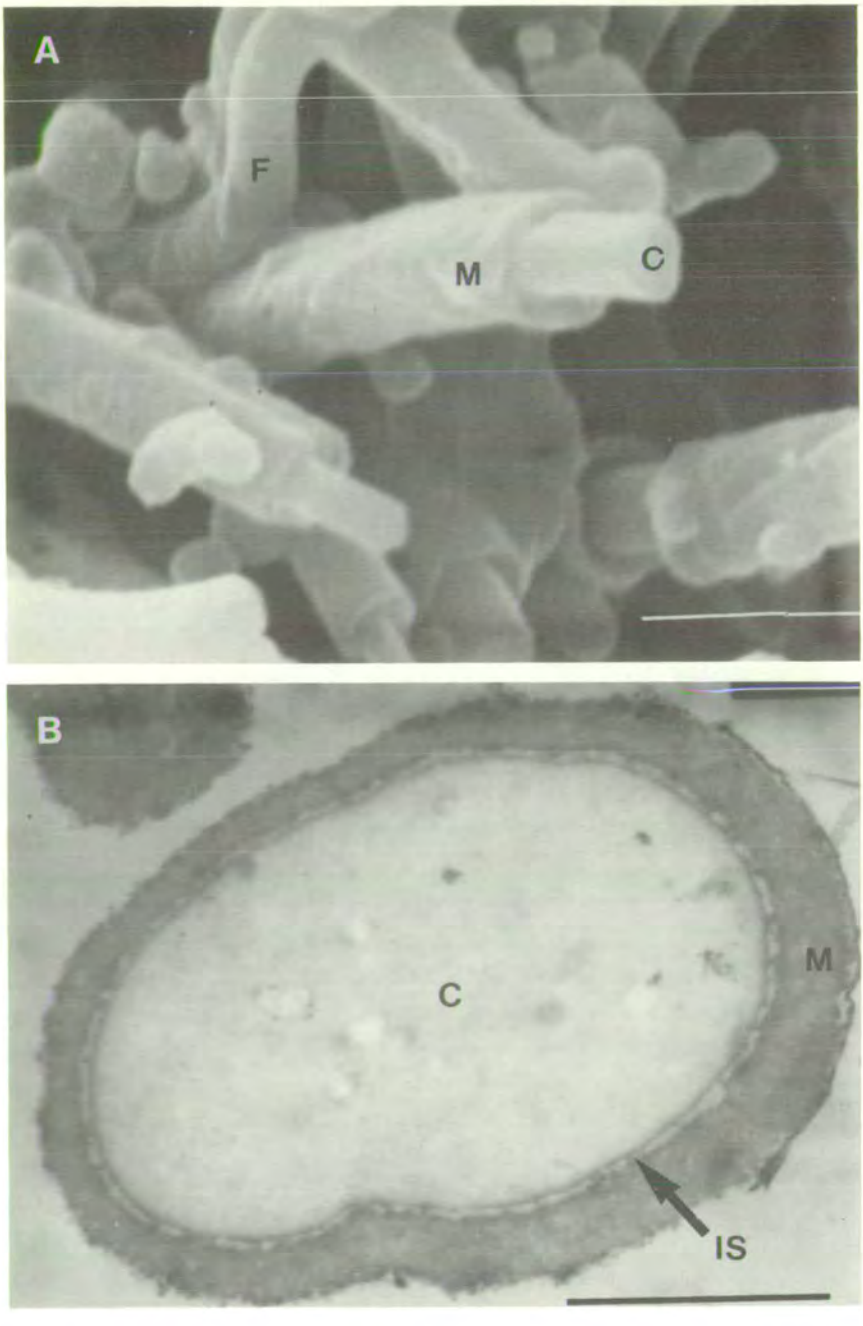
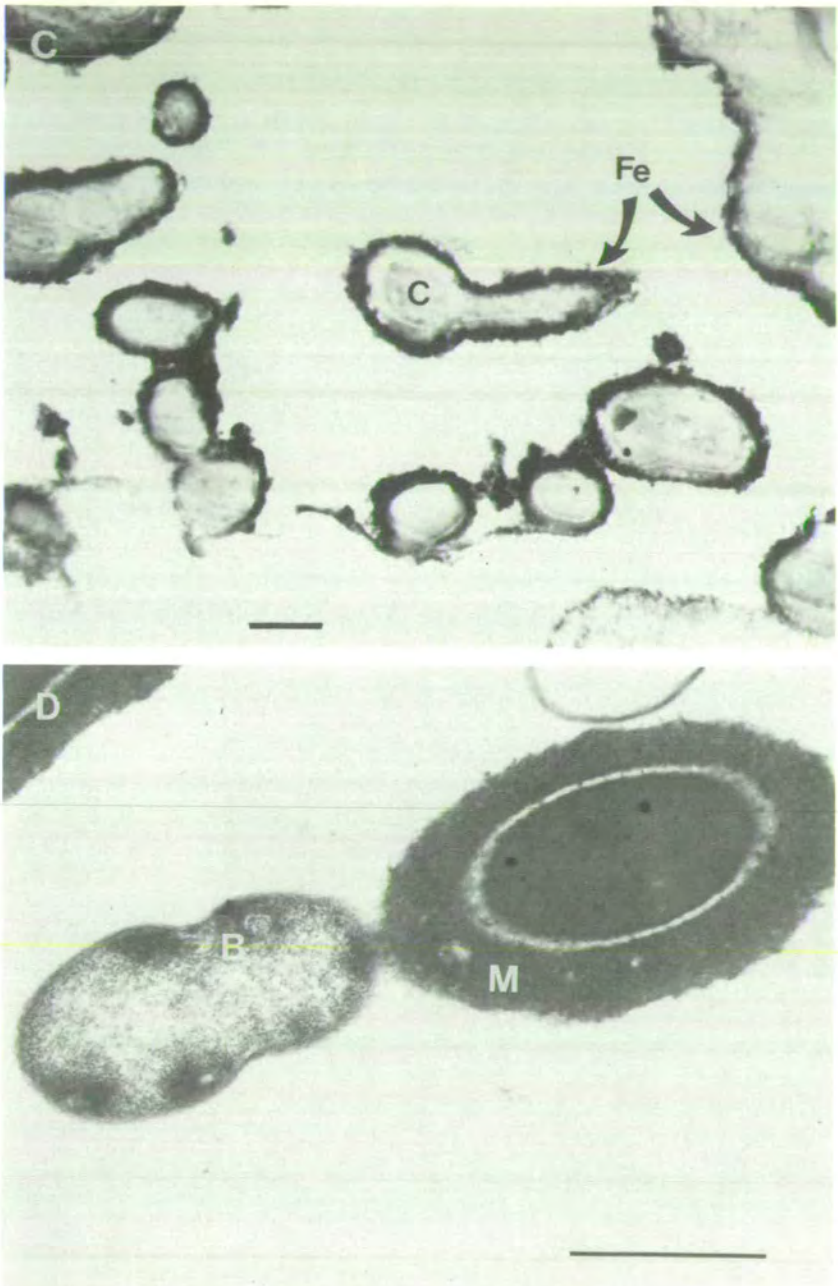


PLATE-FIG. 1.—A. The fibres (F), medulla (M) and core (C) of the shell membranes of the hen's egg shell as seen in scanning electron microscopy; bar marker = 1·00 μm. B. A transverse section of a fibre of the shell membranes of the hen's egg examined with transmission electron microscopy: C, core; M, medulla and IS intrafibrial space; bar marker = 1·00 μm. C. A transverse section of shell membranes of hen's egg treated with colloidal iron and examined with transmission electron microscopy: C, core and Fe, iron-impregnated medulla; bar marker = 1·00 μm. D. A transverse section of shell membranes of hen's egg showing a bacterium (B) adhering to the mantle of the shell membrane (M); bar marker = 1·00 μm. (PLATE-FIG. 1.—continued)



(PLATE-FIG. 1.—continued)

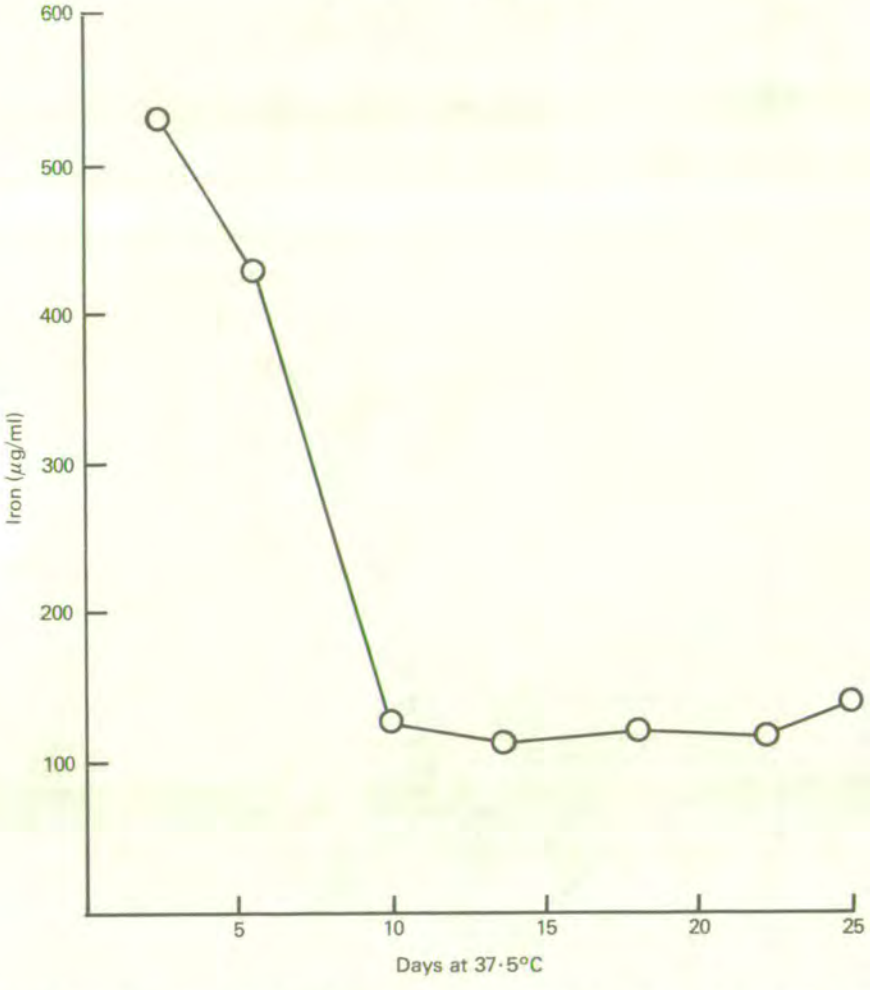


FIG. 1.—Changes in concentration of iron placed on inner shell membrane of incubating infertile eggs. Iron solution (0·1 ml of 6 mg $Fe(NH_4)_2(SO_4)_2.6H_2O$/ml water) was placed on the outer surface of the inner shell membrane of the air space of hens' eggs which were stored at 37·5 °C. At various times, 12 eggs were taken and their inner membranes of the air space excised and hydrolysed in Analar ammonium persulphate (20 g/l) and assayed for iron.

When considered in the context of the extremely low degrees of iron contamination that Garibaldi and Bayne (1960) found to be the cause of enhanced rates and incidence of rotting in washed eggs, the present work indicates that the persistence of iron contamination of membranes would ensure a supply of this element to micro-organisms trapped on or between the fibres. From a practical view point, our observations have identified a potential problem in the methods (Board, 1980) used to control the transmission of pathogens, especially mycoplasma, by incubating eggs. If the antimicrobial agent in which the eggs are caused to contract contained extraneous iron, then this element as well as the antimicrobial agent would be deposited on the

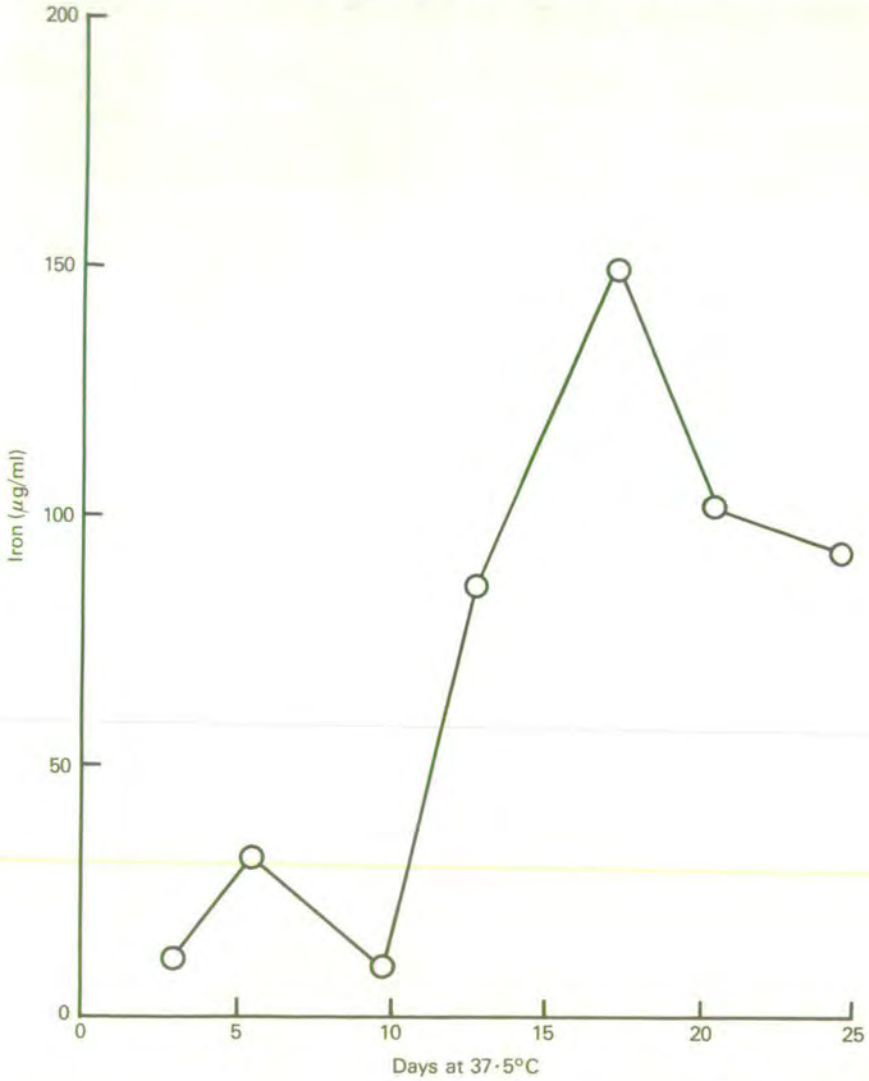


FIG. 2.—Changes in concentration of iron of albumen of incubating infertile eggs following addition of iron to the membranes. Treatment as in Fig. 1, albumen taken, mixed and assayed for iron.

shell membranes. As a consequence, organisms resistant to the antimicrobial agent would have their growth promoted.

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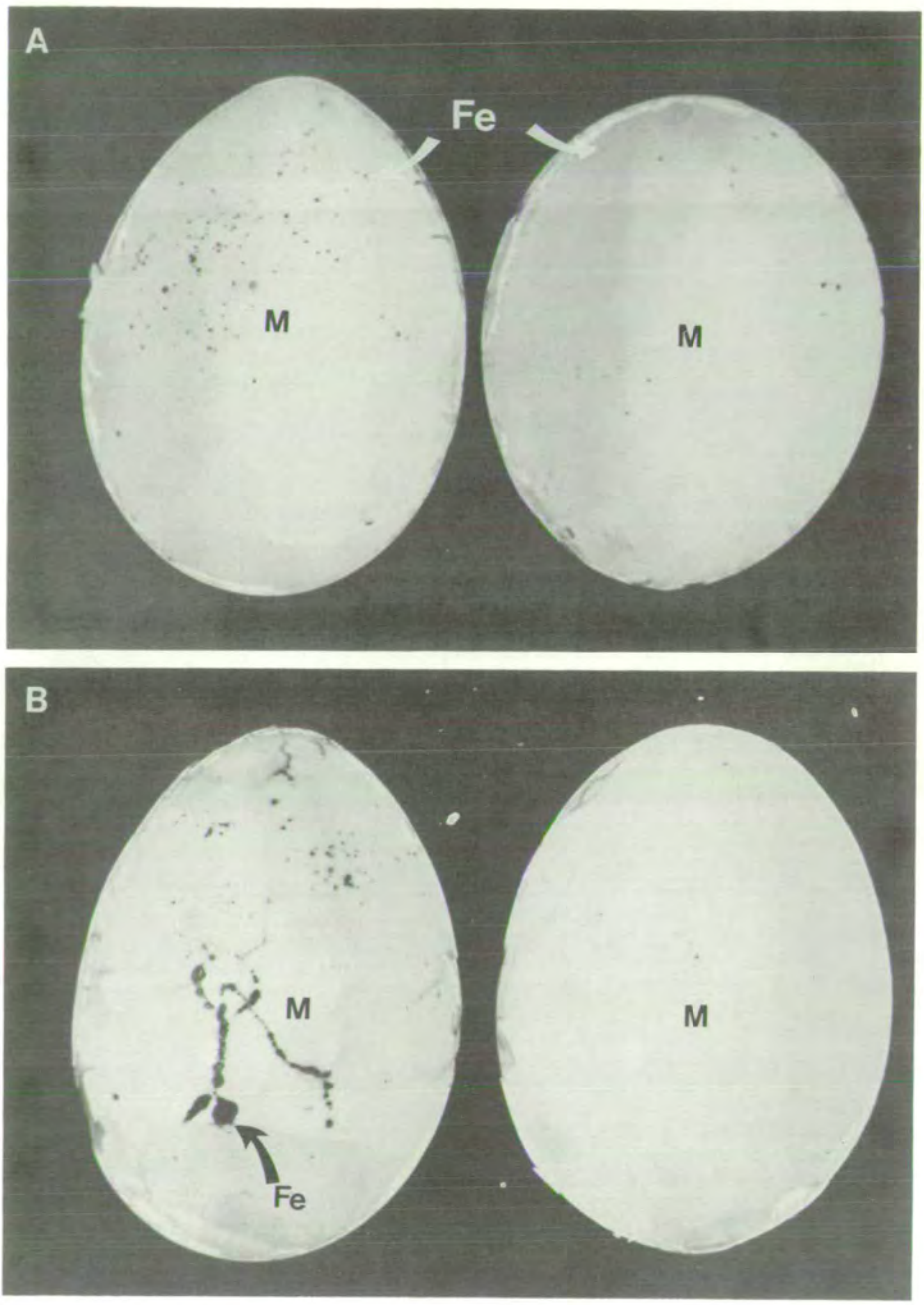
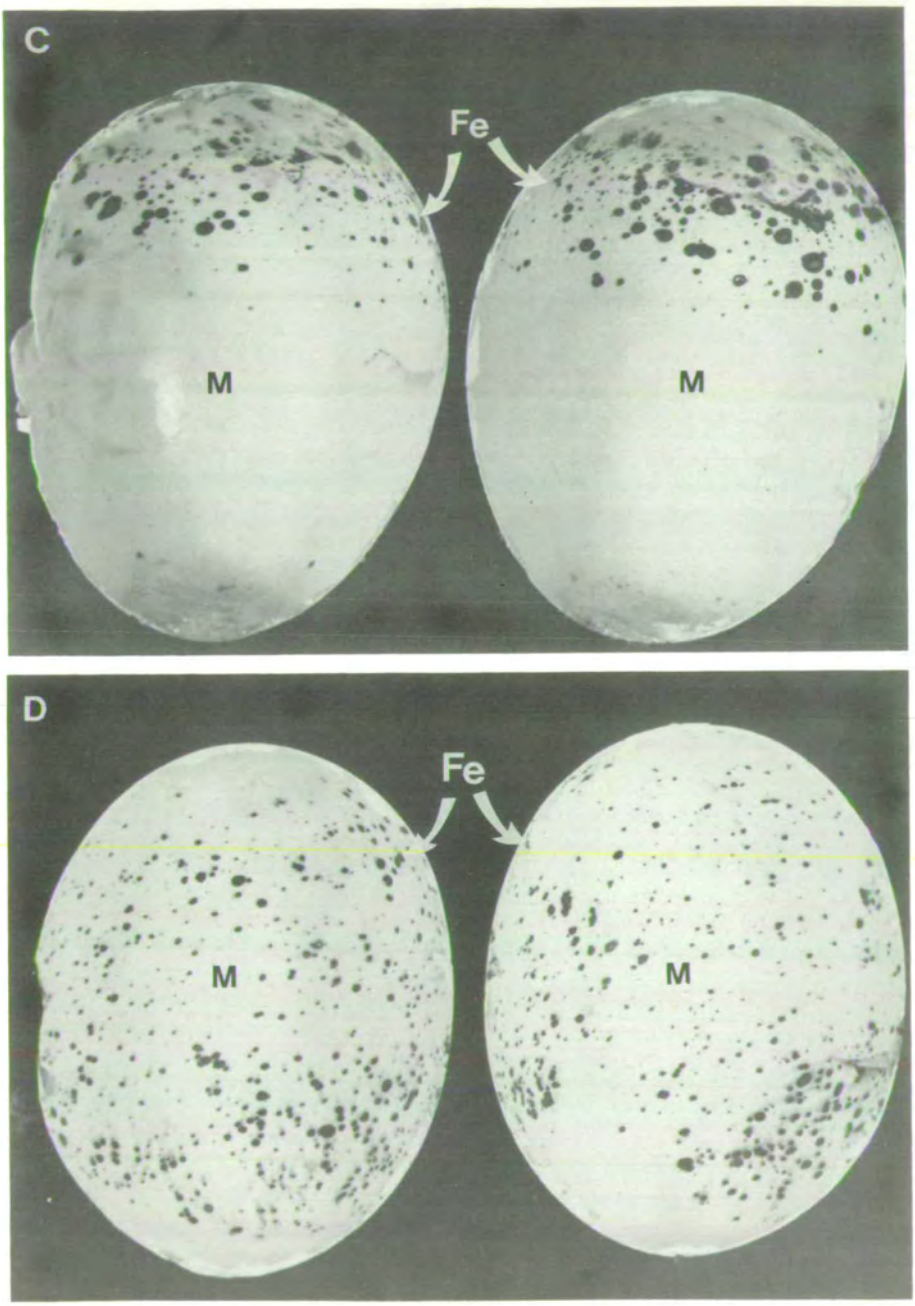


PLATE-FIG. 2.—Hens eggs at 37 °C were immersed for 10 min in ice-cold colloidal iron, the shells cut in half longitudinally, the membranes (M) washed with deionised water and flooded, for 5 min, with potassium ferrocyanide (10 g/l) in 0·1 M HCl, a Prussian blue stain developed (Fe) where iron had penetrated the shell and contaminated the underlying shell membranes. A. An egg with well-formed shell and cuticle. B. An egg of which the shell had been deliberately cracked. C and D. Misshapen eggs with poor cuticles.

(PLATE-FIG. 2.—continued)



(PLATE-FIG. 2.—continued)

REFERENCES

BAKER, J. R. AND BALCH, D. A. (1962). A study of the organic material of the hen's egg shell. *Biochemical Journal*, **82**: 352–361.

BEAN, K. C. AND MACLAURY, D. W. (1959). The bacterial contamination of hatching eggs and methods for its control. *Poultry Science*, **38**: 693–698.

BELLAIRS, R. AND BOYDE, A. (1969). Scanning electron microscopy of the shell membranes of the hen's egg. *Zeitschrift für Zellforschung und Mikroskopische anatomie*, **96**: 237–249.

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *Journal of Applied Bacteriology*, **27**: 350–364.

BOARD, R. G. (1980). The avian eggshell—a resistance network. *Journal of Applied Bacteriology*, **48**: 303–313.

BOARD, R. G. AND AYRES, J. C. (1965). The influence of temperature on bacterial infection of the hen's egg. *Applied Microbiology*, **13**: 358–364.

BOARD, R. G. AND HALLS, N. A. (1973). The cuticle: a barrier to liquid and particle penetration of the shell of the hen's egg. *British Poultry Science*, **14**: 69–97.

BOARD, P. A., HENDEN, L. P. AND BOARD, R. G. (1968). The influence of iron on the course of bacterial infection of the hen's egg. *British Poultry Science*, **9**: 211–215.

BROOKS, J. (1960). Mechanisms of the multiplication of *Pseudomonas* in the hen's egg. *Journal of Applied Bacteriology*, **23**: 499–509.

COLEMAN, J. R. AND TEREPKA, A. R. (1972). Fine structural changes associated with the onset of calcium, sodium and water transport by the chick chorioallantoic membrane. *Journal of Membrane Biology*, **7**: 111–127.

COOKE, A. S. AND BALCH, D. A. (1970). The distribution and carbohydrate composition of the organic matrix in hen egg shell. *British Poultry Science*, **11**: 353–365.

GARIBALDI, J. A. AND BAYNE, H. G. (1960). The effect of iron on *Pseudomonas* spoilage of experimentally infected shell eggs. *Poultry Science*, **39**: 1517–1520.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*a*). Iron and the bacterial spoilage of shell eggs. *Journal of Food Science*, **27**: 57–59.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*b*). The effect of iron on the *Pseudomonas* spoilage of farm washed eggs. *Poultry Science*, **41**: 850–853.

HOFFER, A. P. (1971). The ultrastructure and cytochemistry of the shell membrane-secreting region of the Japanese quail oviduct. *American Journal of Anatomy*, **131**: 253–287.

KAYAR, S. R., SNYDER, G. K., BIRCHARD, G. F. AND BLACK, C. P. (1981). Oxygen permeability of the shell membranes of chicken eggs during development. *Respiration Physiology*, **46**: 209–221.

LEACH, R. M. AND RUCKER, R. B. (1978). Studies on the major protein component of egg shell membranes. *Poultry Science*, **57**: 1151.

LEACH, R. M., RUCKER, R. B. AND VAN DYKE, G. P. (1981). Egg shell membrane protein: A nonelastin desmosine/isodesmosine-containing protein. *Archives of Biochemistry and Biophysics*, **207**: 353–359.

MASSHOFF, W. AND STOLPMANN, H. J. (1961). Licht—und elektronenmikroskopische Untersuchungen an der Schalenhaut und Kalkschale des Huhnereies. *Zeitschrift Für Zellforschung und Mikroskopische anatomie*, **55**: 818–832.

MORAN, T. AND HALE, H. P. (1936). Physics of the hen's egg. 1. Membranes in the egg. *Journal of Experimental Biology*, **13**: 35–41.

NARBAITZ, R. AND TELLIER, P. P. (1974). The differentiation of the chick chorionic epithelium: an experimental study. *Journal of Embryology and Experimental Morphogenesis*, **32**: 365–374.

RINEHART, J. F. AND ABUL-HAJ, S. K. (1951). An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *Archive of Pathology*, **52**: 189.

SIMKISS, K. (1968). The structure and formation of the shell and membranes. In: *Egg Quality—A Study of the Hen's Egg*, pp. 3–25, edit. CARTER, T. C. Edinburgh, Oliver and Boyd.

STARCHER, B. C. AND KING, G. S. (1980). The presence of desmosine and isodesmosine in eggshell membrane protein. *Connective Tissue Research*, **8**: 53–55.

TULLETT, S. G. AND BOARD, R. G. (1976). Oxygen flux across the integument of the avian egg during incubation. *British Poultry Science*, **17**: 441–450.

TUNG, M. A. AND RICHARDS, J. F. (1972). Ultrastructure of the hen's egg shell membranes by electron microscopy. *Journal of Food Science*, **37**: 277–281.

WARING, W. S. AND WERKMAN, C. H. (1942). Growth of bacteria in an iron-free medium. *Archives of Biochemistry*, **1**: 303–310.

WEDRAL, E. M., VADEHRA, D. V. AND BAKER, R. C. (1974). Chemical composition of the cuticle, and the inner and outer shell membranes from eggs of *Gallus gallus*. *Comparative Biochemistry and Physiology*, **47B**: 631–540.

WILLIAMS, J. E. AND WHITTEMORE, A. D. (1967). A method for studying microbial penetration through the outer structures of the avian egg. *Avian Diseases*, **11**: 467–490.

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Review Article: The Course of Microbial Infection of the Hen's Egg

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Contents

1. Introduction

Microbial deterioration of eggs intended for human consumption has attracted considerable attention since the first systematic investigation was conducted by Gayon (1873). In a detailed discussion of the early work Haines (1939) noted that the egg was equipped with physical and chemical defences against microbial infection, and it has been suggested that these have developed to protect the embryo during incubation and that, in the case of market eggs, rotting occurs when the defences are overloaded (Brooks & Taylor, 1955). Although previous reviews (Haines, 1939; Romanoff & Romanoff, 1949; Brooks & Taylor, 1955) provide information on aspects such as the extent of contamination of newly laid eggs, the numbers and types of organisms on the shell, the nature of the flora of rotten eggs and, to a less extent, the chemical and physical basis of the antimicrobial defence, they do not give a clear picture of the events which culminate in the rotting of an egg. Because of its commercial importance this aspect has attracted considerable attention in recent years and the results are discussed in the present review.

2. Contamination of the Egg

(a) *Congenital*

Whether the contents of an egg are free from micro-organisms at the time of laying has remained a vexed question. This can be attributed in part to the technical difficulties associated with sampling the yolk and white under strictly aseptic conditions. Even the most rigorously controlled procedures, using a combination

of detergents, disinfectants and flaming with alcohol, do not ensure sterilization of the shells of all eggs (Gillespie & Scott, 1950; Ayres & Taylor, 1956). Because of their viscosity, the contents cannot be sampled under conditions which can be considered to exclude possible contamination with airborne organisms. Moreover, claims that the shell is easily invaded by micro-organisms in a brief period immediately following laying (Ferdinandov, 1944; Lorenz, Starr, Starr & Ogasawara, 1952; Graves & MacLaury, 1962; Dr. J. Patton, pers. comm.), that the degree of contamination of the contents of fresh eggs is related directly to the porosity of the shell (Kraft, McNally & Brant, 1958) and that qualified bacteriologists recover organisms from fewer eggs than do partly trained technical assistants (Hadley & Caldwell, 1916) cast grave doubts on the exact origin of organisms recovered from the interior of eggs. Some of the earlier findings (Table 1), as indicated by previous reviewers (Haines, 1939; Romanoff & Romanoff, 1949; Brooks & Taylor, 1955),

TABLE 1

Bacterial infection of fresh eggs

No. of eggs examined	% infected in			References
	whites	yolks	yolks and whites	
4,092	—	9·5	NT	Rettger (1913)
2,631	—	8·7	NT	Hadley & Caldwell (1916)
224	2	7	NT	Haines (1938)
651	NT	NT	12	Wolk, McNally & Spicknall (1950)
200	NT	NT	15	Miller & Crawford (1953)

—, No organisms recovered; NT, not tested.

are therefore suspect and they have led to the wide acceptance of the generalization (Brooks & Taylor, 1955) that "roughly 90% of newly laid eggs are free from micro-organisms and the true value may be even higher." This situation is further confused by the observation that the commonest contaminants are micrococci which grow poorly, if at all, at the body temperature of the hen (Hadley & Caldwell, 1916; Haines, 1938; Miller & Crawford, 1953).

In an attempt to resolve this situation, Harry (1963*a*) examined ova obtained from hens which had been killed and dissected in the laboratory and recovered bacteria from 16 of 37 taken from laying hens. Successful recovery of organisms was obtained more frequently with ova which had been held in nutrient broth before plating, thus indicating that relatively few organisms were present in the organs at the time of dissection. A higher incidence of contamination was noted with ova taken from out of lay hens. Lactobacilli and micrococci were the commonest contaminants; organisms resembling *Pasteurella haemolytica* were isolated occasionally. Strains of lactobacilli were recovered from the upper region of the oviduct and again both the incidence and level of contamination were highest with out of lay hens. This may have a physiological basis: the active secretion of egg white

proteins which have antimicrobial properties probably plays an important role in ridding the oviduct of chance contaminants (Horowitz, 1903; Haines, 1939). Harry (1963*a*), in agreement with Rettger (1913), found that the heaviest contamination of the oviduct occurred in the cloacal region, and that the microflora was dominated by micrococci, enterococci and coli-aerogenes organisms.

These observations suggest that the ovaries of healthy hens can become contaminated, but the small number of samples examined to date prevents an estimate of the possible incidence of contamination of newly laid eggs. Moreover, the actual source of the contaminants is not easily determined. Under certain conditions they may migrate upwards from the cloaca or be carried up when the oviduct undergoes violent antiperistaltic contraction of the type which causes feathers and small stones to be included in the albumen of otherwise normal eggs (Romanoff & Romanoff, 1949). It is known that organisms placed in the oviduct remain viable for 48–72 h (Horowitz, 1903) and that, with salmonellae, the ovaries can become infected (Rettger, 1913). This route of contamination was considered by Harry (1963*a*) but he favoured the view that blood borne organisms are principally responsible for the contamination of the ova *in vivo*, although little success attended his attempts to recover organisms from the vascular system. There is, however, abundant evidence (Rettger, 1913; May, 1924; Buxton & Gordon, 1947; Gordon & Tucker, 1965) that *Salmonella* spp. pass from the alimentary canal *via* the blood to the ovaries, but there is no conclusive evidence of such migration by nonpathogens.

Miles & Halnan (1937) were unable to promote rotting of eggs by feeding or injecting hens with *Proteus melanovogenes*, and the design of the experiments of Bennetts (1931) and Platt (1936) does not allow an unqualified acceptance of their conclusions that the disorders of the eggs which they examined were due to contamination *in vivo*—contamination with organisms present on nest litter etc. cannot be excluded.

Contamination during the formation of an egg with organisms capable of causing deterioration of the yolk and white has not been demonstrated therefore with certainty. A probability that rot producing organisms are of extragenital origin is supported by the observation that less than 1% of naturally clean eggs rot during prolonged storage (Brooks & Taylor, 1955).

(b) *Extragenital*

The evidence discussed above suggests that the shells of a few eggs may be contaminated when passing along the oviduct, particularly in the cloacal region, but there is little direct evidence to this effect. Stuart & McNally (1943) did not recover organisms from the shells of 6 eggs which had been dissected from the oviduct, although 2 out of 6 shells were contaminated when the eggs were collected aseptically at the time of oviposition. This indicates that the main contamination of the shell occurs after laying. Many investigations (Table 2) have been concerned with the number of micro-organisms on the shell and averages in the range $9{\cdot}5$–$3{,}100 \times 10^3$/shell have been reported. The extent of contamination appears to be a function of (1) the cleanliness of the nest boxes, the heaviest being associated with dirty nesting litter (Harry, 1963*b*), and (2) the manner in which the eggs are handled after laying. Thus

TABLE 2

Levels of microbial contamination of the shell of the hen's egg

Source of supply	No. of eggs examined	Grade and/or treatment of eggs	No. of micro-organisms/shell		Country	Reference
			Mean	Range		
Farm	36	Handled with gloved hands	$9{\cdot}5\times10^{3}$	*	Canada	Rosser (1942)
Batteries	25	*	$2{\cdot}5\times10^{4}$	$2{\cdot}5\times10^{3}$–$8{\cdot}1\times10^{4}$	England	Harry (1963*b*)
Experimental farm	*	*	$6{\cdot}3\times10^{4}$	$1{\cdot}0\times10^{4}$–$1{\cdot}0\times10^{6}$	U.S.A.	Forsythe *et al.* (1953)
Packing station	72	*	$7{\cdot}0\times10^{4}$	*	Canada	Rosser (1942)
Farm	36	*	$1{\cdot}0\times10^{5}$	*	Canada	Rosser (1942)
Shops and farms	130	*	$1{\cdot}3\times10^{5}$	*	England	Haines (1938)
Packing station	73	Clean: Grade A	$2{\cdot}2\times10^{5}$	$3{\cdot}0\times10^{2}$–$1{\cdot}0\times10^{7}$	U.S.A.	Board *et al.* (1964)
Deep litter	25	*	$3{\cdot}5\times10^{5}$	$6{\cdot}2\times10^{3}$–$2{\cdot}4\times10^{6}$	England	Harry (1963*b*)
Packing station	77	Lightly soiled: Grade B	$9{\cdot}7\times10^{5}$	$1{\cdot}0\times10^{3}$–$1{\cdot}9\times10^{7}$	U.S.A.	Board *et al.* (1964)
Deep litter	96	Clean and lightly soiled	$3{\cdot}1\times10^{6}$	$5{\cdot}0\times10^{1}$–$1{\cdot}0\times10^{7}$	Scotland	Board & Wilson (1965)

* Details not given in original report.

TABLE 3

Types of micro-organisms recovered from the shell of the hen's egg

Type of organism	Incidence (%) of organisms on eggs of different conditions from						
	Shops and farms§§	Egg breaking plants‡			Packing station§		
		Clean	Lightly soiled	Heavily soiled	Clean	Lightly soiled	Cracked etc.
Streptococci	—	8	5	—	—	—	—
Staphylococci	5	30	—	—	9	5	11
Micrococci	18	23	20	—	37	52	42
Sarcinae	2	20	—	—	—	—	—
Arthrobacters	—	—	—	—	5	13	10
Bacilli	30	—	13	5	—	2·5	—
Pseudomonads	6	—	—	—	—	—	—
pigmented	—	—	—	—	1·5	2·5	1
non pigmented	—	—	—	—	21	10	23
Achromobacters	19	—	—	—	1·5	2	1
Alcaligenes	—	—	—	—	—	2	—
Flavobacteria	3	—	—	—	—	—	—
Cytophaga	—	—	—	—	—	1	—
Escherichiae	4	12	7	2	4·5	7	2
Aerobacters	1	7	—	3	6	0·5	2
Aeromonads	—	—	—	—	1	—	2
Proteus	1	—	20	20	—	—	—
Serratiae	—	—	20	50	—	—	—
Moulds	7	—	10	20	—	—	—
Unclassified	—	—	—	—	12†	5†	6†
No. of organisms studied	100	*	*	*	130	164	126

* Details not given; † aerobic Gram negative bacteria.
§§ Haines (1938); ‡ Zagaevsky & Lutikova (1944); § Board *et al.* (1964).

Rosser (1942) found the level of contamination on the shells of eggs handled with gloved hands was less than when the eggs were handled normally. The microflora of the shell is dominated by Gram positive bacteria (Table 3) derived almost certainly from dust, soil and faeces (Haines, 1939; Zagaevsky & Lutikova, 1944; Board, Ayres, Kraft & Forsythe, 1964).

Although Gram positive bacteria are the dominant contaminants of the shell a mixed infection of Gram negative bacteria appears to be typical of rotten and tainted eggs (Table 4). The properties and classification of these organisms have been discussed in detail elsewhere (Board, 1965*b*) and it will suffice to note that the common contaminants of rotten eggs belong to the genera *Alcaligenes*, *Achromobacter*, *Pseudomonas*, *Serratia*, *Cloaca*, *Proteus*, *Citrobacter* and *Aeromonas*. It is noteworthy also that Gram negative bacteria are the organisms which have been detected most frequently in the contents of incubated eggs (Harry, 1957; Pathak, Singh & Tangri, 1960; Reid, Macy, Boyd, Kleckner & Schmittle, 1961). This evidence indicates

TABLE 4

Types of organisms recovered from the contents of rotten or tainted eggs

Organisms	Occurrence in				
	Rotten eggs reported by				Tainted eggs reported by
	Haines 1938)	Alford *et al.* (1950)	Florian & Trussell (1957)	Board (1965*b*)	Richard & Mohler (1950)
Coli-aerogenes	+	+	+	+	+
Proteus	+	+	+	+	−
Aeromonads	−	−	+	+	−
Pseudomonads	+	+	+	+	+
Alcaligenes	+	+	+	+	+
Achromobacters	+	+	+	+	+
Gram positive bacteria	−	−	−	±	±

+, Strains isolated on many occasions; ±, strains isolated occasionally; −, strains not isolated.

that Gram negative bacteria are better equipped than Gram positive ones to overcome the antimicrobial defences of the egg. The reason for this will be considered later.

3. Course of Infection

When reviewing the early literature on this topic, Gillespie & Scott (1950) concluded that three major events lead up to the rotting of an egg. These are (1) microbial penetration of the shell, (2) growth on and possible digestion of the shell membranes and (3) infection of the albumen. The general validity of this concept has been confirmed by recent investigations.

(a) *Penetration of the shell*

The early work on the structure of the hen's egg shell has been reviewed by Stewart (1935), Romanoff & Romanoff (1949) and Simkiss (1961). It is 241–371 μ thick (Tyler, 1961*a*) and is perforated with 7,000–17,000 pores (Tyler, 1953), the diameters of which are in the range 9–35 μ (Romanoff & Romanoff, 1949; Tyler, 1956). The shape of the pore is shown in Fig. 1. It is partially filled with proteinaceous material—the cuticular plug of Simkiss (1961). Yeasts and bacteria can be sucked through shells from which the shell membranes have been removed (Haines & Moron, 1940; Garibaldi & Stokes, 1958). In such experiments the organisms were drawn from the inside of the shell, a direction opposite to that taken during invasion of intact eggs and one which might be suspected of diminishing the effect of the cuticular plug.

Ferdinandov (1944) claimed that the shell is susceptible to bacterial penetration within a very short time after laying. This was based on the assumption that the yolk and white contract on cooling, thereby causing organisms to be sucked through

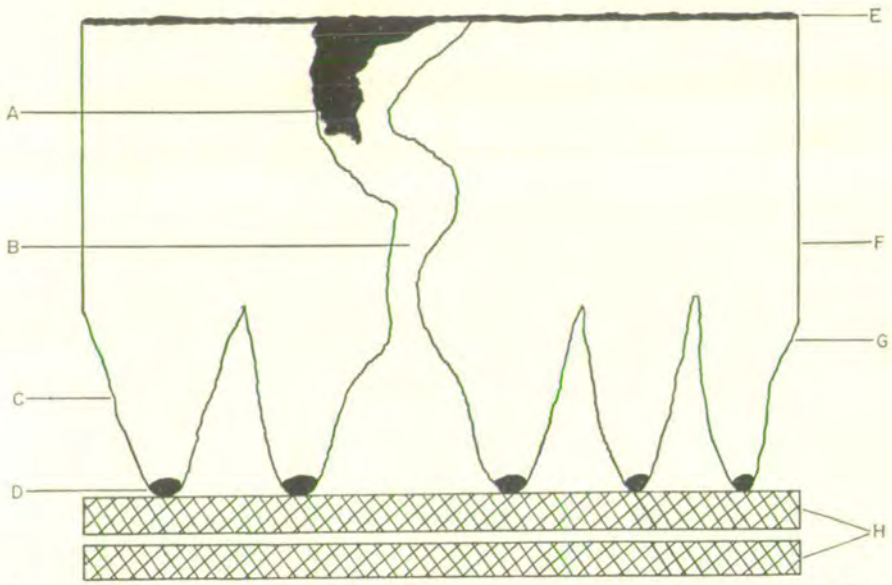

Fig. 1. A conventional drawing of a radial section of an egg shell. A, Cuticular plug; B, pore; C, mammillary knob; D, mammillary core; E, cuticle; F, spongy matrix; G, mammillary matrix; H, shell membranes.

pores which are still moist and so not effectively blocked. It is pertinent to note that a high incidence of rotting occurs during the storage of eggs gathered from nests in which the litter was contaminated deliberately with large numbers of rot-producing bacteria (Lorenz, Starr, Starr & Ogasawara, 1952; Dr. J. Patton, pers. comm.). This mode of infection would not appear to be of any commercial significance, however, since it has been established (Brooks & Taylor, 1955) that less than 1% of nest clean eggs rot during prolonged storage.

There is a dearth of evidence on the exact process whereby micro-organisms traverse the pores of the shell. Our knowledge is based on the evidence of experiments in which eggs are immersed in a suspension of rot-producing bacteria and the incidence of rotting is used as an index of the efficiency of the shell as a barrier to penetration. Thus Haines & Moran (1940) found that effective penetration occurred in about 18% of naturally clean eggs which had been immersed for a short time in a suspension of pseudomonads having the same temperature as the eggs. Under such conditions water will be drawn into the pores by capillary attraction and bacteria will be lodged in the shell membranes. As far as can be ascertained the possible role of osmotic forces acting across the semipermeable shell membranes has not been considered, although it is known that these can cause the rupture of the shell membranes when eggs, from which a small piece of shell has been taken, are placed in water (Lifshitz, Baker & Naylor, 1964). A higher incidence of contamination (Gordon & Tucker, 1954) and a greatly increased incidence of rotting (Haines, 1938; Haines & Moran, 1940) occur when the shells are rubbed with sand paper or steel wool before the eggs are placed in a bacterial suspension. This

indicates that cuticular debris in the orifice of the pores play an important role in preventing microbial penetration of the shell. The removal of this material results in a marked increase in the loss of water from the albumen (Bryant & Sharp, 1934; Marshall & Cruickshank, 1938; Tyler, 1945).

A high incidence of rotting occurs in eggs which are warm when dipped in a cold suspension of rot-producing bacteria (Haines & Moran, 1940). This phenomenon was investigated by Bean & MacLaury (1959), using *Str. faecalis*, *Pr. morganii*, *Pr. vulgaris* and *Ps. aeruginosa*. Viable organisms were recovered from 90–100% of the inner surfaces of the shells and from 5–90% of the shell membranes of eggs when examined immediately after immersion. In an attempt to get further information Mr. G. Torrey and the author (unpublished observations) used a modification of the method of Rievel (1939). Warm (30°) eggs were immersed in water containing ice and *Serr. marcescens* (*c.* $1{\cdot}0\times10^6$/ml) for 10 min and then removed and dried under a fan. The shell at the pointed end of each egg was cut with a carborundum disc, the underlying shell membrane severed with a sterile scalpel and the contents discarded. The inside of the shell was then flushed with sterile water and filled with glycerol asparagine agar (Sullivan, 1905). After sealing the hole in the shell with paraffin wax the eggs were laid on Keyes trays and inspected daily with transmitted light during incubation at 30°. No growth occurred within the agar but discrete colonies to a maximum of 10/egg developed on all of the membranes. The colonies developed under or in close proximity to pores having an outer orifice sufficiently large to be seen with the naked eye. No growth occurred in eggs which had not been immersed or in others which had been immersed for 10 min in a warm (30°) suspension of *Serr. marcescens*. This evidence indicates that the main portals for the entry of micro-organisms are the 10 or so large pores which are present in the majority of shells, a possibility suggested previously by Bryant & Sharp (1934) and Orel (1959) following their investigations of the effect of washing on the keeping quality of eggs.

Under the conditions discussed above it is generally assumed that the yolk and white contract more than does the shell, thus causing water and micro-organisms to be drawn into the pores. On the assumption that in such experiments the incidence of rotting during the storage of eggs provides a true index of penetration of the shell, the following factors are considered to play an important part in microbial invasion of the shell and to be of particular importance in the cleaning of market eggs: (1) the temperature differential between the egg and the bacterial suspension, the incidence of rotting being directly proportional to the difference in temperature in the range 6–21° (Lorenz *et al.*, 1952; Brant & Starr, 1962); (2) the number of organisms in the suspension (Lorenz *et al.*, 1952; Stokes, Osborne & Bayne, 1956; Brant & Starr, 1962; Hartung & Stadelman, 1963); (3) the period of immersion (Brant & Starr, 1962; Hartung & Stadelman, 1962); (4) the thickness of the shell, thin shells offering less resistance than thick ones (Orel, 1959); (5) the treatment of the shell before immersion, the incidence of rotting being increased when the shells are rubbed with materials such as cheesecloth, sandpaper or steel wool.

The mere wiping of a shell with a cloth or brush moistened with a bacterial culture will result in contamination of the egg's contents (Stuart & McNally, 1943; Wilson,

1945; Buxton & Gordon, 1947; Lancaster & Crabb, 1953), but it is not clear whether this is due to water being drawn into pores by capillary attraction or to it being forced into the orifice of the pores (Fig. 1). It has been claimed that microbial invasion of the shell occurs when condensation ('sweating') occurs on the shells of cold eggs (Fromm & Margoff, 1958) but again the actual cause of penetration is uncertain. In other experiments it has been established that fungal hyphae can grow through the pores (Weston & Halnan, 1927) and deposit bacteria on the shell membranes (Zagaevsky & Lutikova, 1944).

No success has attended attempts to demonstrate bacterial multiplication in the shells of eggs held under normal conditions of storage (Haines, 1938; Forsythe, Ayres & Radlo, 1953; Board *et al.*, 1964). With hatching eggs it has been reported (Lancaster & Crabb, 1953; Magwood, 1964) that there is a rapid death of bacteria placed on the surface of the shell provided that the latter is not dirty and the eggs are not held under very humid conditions. From a theoretical standpoint, Gillespie & Scott (1950) concluded that bacterial multiplication would not occur in the greater part of the shell because of the lack of water. On the assumption that the water activity of the shell membranes is 0·995 and that the water vapour pressure gradient in the shell is linear, they considered that a level of available water suitable for the growth of the common rot-producing bacteria (0·98) would extend outwards from the shell membranes for a distance of 34 μ in eggs held at 0° and a relative humidity of 85%. It is of interest to note that Sharp & Stuart (1936) found that moulds did not develop on the shell unless the relative humidity of the store was 90% and good growth did not occur unless the relative humidity was 96% or greater. This evidence indicates that under normal commercial conditions, bacteria have to penetrate at least to the mammillary layer (Fig. 1) before they are in a position to grow and infect the albumen.

(b) *Penetration and colonization of the shell membranes*

Many investigators have noted a lag of up to 20 days between penetration of the shells of newly laid eggs and the occurrence of large numbers of organisms or of macroscopic changes in the albumen (Zagaevsky & Lutikova, 1944; Gillespie & Scott, 1950; Bigland & Papas, 1953; Miller & Crawford, 1953; Elliott, 1954; Stokes *et al.*, 1956; Orel, 1959; Fromm & Monroe, 1960; Garibaldi & Bayne, 1960). This has been attributed to the shell membranes providing a mechanical barrier against microbial invasion (Kraft, Elliott & Brant, 1958; Hartung & Stadelman, 1962) or to a combination of this property and antimicrobial substances in the membranes (Brooks & Taylor, 1955). Recent investigations (Brooks, 1960; Board, 1964; Board & Ayres, 1965) have indicated that the lag is primarily a reflection of the antimicrobial nature of the albumen and that the shell membranes play only a minor role in restraining microbial invasion of the albumen.

Penetration

The outer and inner shell membranes are 50–75 μ and 15–17 μ thick, respectively (Tyler, 1961*b*; Lifshitz & Baker, 1964). Each membrane is formed of laminae composed of networks of fibres, chiefly of keratin and mucin, while the interstices are filled with albumen (Romanoff & Romanoff, 1949). The fibres of the innermost

layers are much finer and more closely packed than those in the outer layers (Masshoff & Stolpman, 1961).

The concept that the shell membranes behave as bacterial filters arose from the investigations of Haines & Moran (1940). Using eggs in which the contents had been replaced with a suspension of bacteria, they were unable to recover organisms from fluid which had been sucked through the shell. This was confirmed in the later investigations of Walden, Allen & Trussell (1956) and Garibaldi & Stokes (1958) although these revealed that the fluid contained organisms when suction was not applied until 18–24 h following the introduction of the bacterial suspension to the inside of the egg. The process which results in the penetration of the shell membranes is not known but it does not appear to be due to the digestion of the membranes by bacterial proteases (Garibaldi & Stokes, 1958; Board, 1965*b*). Further evidence that the shell membranes do not impose an effective barrier to bacterial invasion of the albumen came from attempts to recover organisms from the albumen following the seeding of the air cell. From this site the bacteria must pass through the inner shell membrane, and this has a much greater resistance to penetration than the outer membrane (Lifshitz *et al.*, 1964). In the original work (Miller & Crawford, 1953; Elliott, 1954) organisms were not recovered from the albumen until 4–6 days after inoculating the air cell. Subsequent investigations have shown that the membrane is quickly invaded when large inocula are used (Brooks, 1960; Board, 1964) especially when the eggs are held at 37° (Board & Ayres, 1965). Similarly, experiments with pieces of the shell membrane *in vitro* (Hartung & Stadelman, 1962) have shown that it is quickly breached when challenged with large numbers of organisms. Thus it would appear that the lag noted at the beginning of this section cannot be attributed to the shell membrane imposing an effective barrier to bacterial penetration.

Colonization

At one time it was considered (Brooks & Taylor, 1955) that the membranes contain antimicrobial substances which suppress the growth of organisms which have penetrated through the shell and this has been confirmed with certain Gram positive bacteria. Korotkova (1957) detected lysozyme in the shell membranes and considered that this played an important part in the defence of the developing embryo. This might also account for the low incidence of Gram positive bacteria in the contents of rotten eggs (Table 4). The belief that the membranes may also destroy Gram negative bacteria arose from certain observations of Stuart & McNally (1943) but later investigators have shown that when the membranes are suspended in a nontoxic solution of mineral salts (Elliott & Brant, 1957) good growth of the common contaminants of rotten eggs occurs either when intact (Board, 1965*a*) or comminuted membranes (Stokes & Osborne, 1956; Garibaldi & Bayne, 1958) are used.

Direct evidence of the process of colonization of the shell membranes comes from experiments in which bacteriological and chemical methods were used to study the behaviour of organisms placed on the inner membrane of the air cell. The majority of investigators incubated the eggs in the range 20–30°, i.e. commercial 'room temperature', and all of them have used the common contaminants of rotten eggs.

Room temperature. A primary phase of multiplication occurs in the 2–4 days following inoculation of the air cell of newly laid eggs (Brooks, 1960; Board, 1964). With small inocula this results in a relatively small increase in the size of the population in the inner membrane, but when large inocula are used the albumen is also invaded but there is no multiplication. The small increase in the number of organisms in the inner membrane is surprising in view of the amount of multiplication which occurs in a suspension of shell membranes (Stokes & Osborne, 1956; Elliott & Brant, 1957; Garibaldi & Stokes, 1958). Brooks (1960) was of the opinion that the growth of bacteria on the shell membranes *in situ* was restricted because of a deficiency of available iron and this view received support from the demonstration (Board, 1964) that considerable bacterial multiplication occurs immediately following the seeding of the air cell with organisms suspended in a weak solution of ferrous sulphate. This evidence suggests that in the primary phase multiplication is limited by the action of the chelating agent, conalbumin. Although this substance has not been shown to be present in the shell membranes, bacterial multiplication is hindered when a dialysis tube containing conalbumin is placed in freshly inoculated nutrient broth (Feeney & Nagy, 1952). Thus it would appear that the antimicrobial properties of the albumen are primarily responsible for confining multiplication to the shell membranes.

The period of decline which follows the primary phase of multiplication results presumably from the death of organisms in the membranes or their migration to the albumen. Later a secondary phase of multiplication occurs. Its induction has been attributed (Brooks, 1960) to a spontaneous change in the properties of the shell membranes. Other workers (Elliott & Brant, 1957; Hartung & Stadelman, 1963) have also suggested that the properties of the membranes change during storage, although the physical or chemical basis of the changes were not elucidated. Sharp & Whitaker (1927) suggested that the multiplication of organisms which invade the albumen does not occur until they have made contact with the yolk, and recent investigations (Board, 1964; Board & Ayres, 1965) have established that a renewed multiplication occurs only when the yolk makes contact with the inner shell membrane. At this time there is a gross infection of the albumen and the yolk (Brooks, 1960), a marked reduction occurs in the amount of glucose in the albumen, the pH of the white moves towards neutrality, and, in eggs inoculated with chromogenic or proteolytic organisms, macroscopic changes appear in the albumen and yolk (Board, 1964). The evidence suggests that the duration of the lag in bacterial growth after the organisms have penetrated the shell is determined by the rate at which the yolk moves towards the shell membrane.

Other temperatures. Two distinct phases of multiplication do not appear to occur in eggs inoculated with pseudomonads and held at 10° (Board & Ayres, 1965). Slow multiplication of the organisms in the shell membranes occurred in the 14 days following inoculation, during which time there also appeared to be a gradual increase in the number of organisms in the albumen. In the 14–21 day period many of the eggs showed a marked increase in the number of organisms in the shell membranes and albumen and the whites were stained with a fluorescent green pigment. These changes occurred without any evidence of a union of the yolk

and shell membranes and it was presumed that they were initiated by organisms which had reached the surface of the yolk. Only one phase of multiplication has been reported (Board & Ayres, 1965) in eggs inoculated with *Ser. marcescens* and held at 37°. This occurred when the yolk made contact with the inoculated shell membrane. These observations indicate that temperature may play an important role in the coordination of the antimicrobial defence of the egg.

(c) *Bacterial growth in the albumen*

The evidence reviewed above indicates that a distinction should be drawn between albumen containing resting or moribund organisms and that containing actively dividing organisms. According to Haines (1939) the notion that the white of eggs contains substances inimical to bacterial growth originated from investigations conducted in the decade 1880–1890. In the ensuing years our knowledge of the nature of these has been founded on evidence coming from two different but complimentary methods of investigation. In one it has been demonstrated that several of the constituents, chiefly proteins, of egg white possess specific biological activities and it has been inferred that these play a role in the defence of the egg. A list of such substances together with their biological activities is given in Table 5. In the

TABLE 5

Antimicrobial components of the albumen of the hen's egg

Component	Action	Investigators
Lysozyme	Lysis of cells walls of Gram positive bacteria	Laschtschenko (1909), Fleming (1922)
	Flocculation of bacterial cells	Friedberger & Hoder (1932)
Conalbumin	Chelation of iron	Schade & Caroline (1944); Feeney & Nagy (1952); Garibaldi (1960)
Ovomucoid	Inhibition of trypsin	Balls & Swenson (1934); Lineweaver & Murray (1947)
Avidin	Combination with biotin	Eakin, Snell & Williams (1940); Woolley & Longsworth (1942); Baumgärtner (1957)
Riboflavin	Chelation of cations	Feeney & Nagy (1952)
Uncharacterized proteins		
A	Inhibition of trypsin and chemotrypsin	Rhodes, Bennett & Feeney (1960)
B	Inhibition of fungal protease	Masushima (1958)
C	Combination with riboflavin	Rhodes, Bennett & Feeney (1959)
D	Combination with vitamin B_6	Evans, Butts & Davidson (1951)

other method of investigation, albumen *in vitro* was inoculated and, by modifications such as the addition of nutrients or the adjustment of pH, attempts were made to attain growth rates of the same magnitude as those given by the test organism in a nutritionally adequate medium. As a result of improvements in the methods for the isolation, purification and identification of egg white proteins (Baker & Manwell, 1962), the second approach has allowed the physical and chemical basis

of the toxicity of the white to be defined and made it possible to assess the relative importance of many of the components shown in Table 5.

The first incisive study of this aspect of egg microbiology can be considered to be that of Laschtschenko (1909). He observed lysis of *B. subtilis* seeded into egg white and noted that this did not occur with egg white which had been held at 65–70° for 30 min. The enzymic nature of this phenomenon was confirmed by Fleming (1922) and he proposed the name lysozyme for the lytic agent. It is now generally agreed that lysozyme plays a major role in the defence of the egg against Gram positive bacteria. It has been shown also that both sensitive and insensitive organisms are agglutinated by high dilutions of lysozyme (Friedberger & Hoder, 1932) and this has been attributed to the basic properties of the molecule (Salton, 1957). It is not known whether this secondary attribute of lysozyme plays a role in impeding the movement of bacteria in the albumen.

The observation (Peter & Healey, 1925) that in the week following laying the reaction of the albumen changes from pH 7·5 to 9·5 led Sharp & Whitaker (1927) to investigate the influence of the hydrogen ion concentration of the albumen on the growth of bacteria. Using common contaminants of rotten eggs and an incubation temperature of 37°, they found that significant multiplication occurred in the 6 h following inoculation of albumen adjusted to pH 6–8 but that the organisms were killed when the albumen was adjusted to pH 9–10. These observations provide a possible interpretation of the reports that freshly laid eggs are less toxic than stored eggs (Turro, 1902; Lutsky & Bell, 1953) and that there was no toxicity associated with the albumen of eggs which were coated with paraffin wax immediately following laying (Parascandole, 1893), a treatment which prevents CO_2 diffusion and the concomitant shift in pH (Sharp & Powell, 1931). Sharp & Whitaker (1927) found that rapid multiplication occurred in thermally or chemically denatured albumen at pH 9·4 and in albumen which had been dialyzed. This led them to conclude that pH alone plays only a minor role in the defence of the albumen against infection by Gram negative bacteria. The important component appeared to be a thermolabile substance the activity of which was influenced by the pH. The loss of toxicity following dialysis of the albumen led Sharp & Whitaker to consider that this substance was not a protein.

The exact nature of this substance remained a mystery until the work of Schade & Caroline (1944) who worked with *Sh. dysenteriae*, *Staph. aureus*, *E. coli* and *S. cerevisiae*. They noted growth inhibition in nutrient broth supplemented with egg white when the mixture was adjusted to pH 7·4 or greater but not when it was adjusted to pH 5·8 or less. Of 10 vitamins and 31 elements tested, iron alone overcame the inhibition. The iron binding, inhibitory substance was isolated from egg white by Alderton, Ward & Fevold (1946) and they identified it with conalbumin, an albuminous protein which was originally isolated from eggs by Osborne & Campbell (1900). This organic ligand, which differs from the serum albumin, transferrin, only in the composition of its carbohydrate prosthetic group (Williams, 1962) accounts for 10% of the total egg white solids (Longsworth, Cannan & MacInnes, 1940; Alderton *et al.*, 1946); it is uniformly distributed throughout the white (Feeney, Ducay, Silva & MacDonnell, 1952); its sequestering power is not

detectably reduced by short term storage of eggs (Feeney *et al.*, 1952) and its chelating potential would not appear to be exhausted by iron that diffuses from the shell during storage of eggs for short periods (Schaible, Bandemer & Davidson, 1946). Conalbumin forms a chromogenic (salmon-pink) complex with ferric ions (Fraenkel-Conrat & Feeney, 1950; Warner & Weber, 1951) and, although the active groups have not been identified with certainty, the hydroxyl groups of tyrosin have been implicated (Warner & Weber, 1953). It is pertinent to note also that the conalbumin-iron complex is more resistant than conalbumin alone to enzymic digestion, thermal denaturation and in other ways (Fuller & Briggs, 1956; Azari & Feeney, 1958, 1961).

When pure preparations of conalbumin have been used to inhibit microbial growth, it has been established that they must be present in stoichiometric excess of the iron found in the medium by chemical analysis (Fraenkel-Conrat & Feeney, 1950; Feeney & Nagy, 1952; Schade, 1958). Feeney and his collaborators found that inhibition expressed itself by an increase in the lag phase of growth and a decreased rate of multiplication once growth had begun. They also found that different organisms showed different degrees of inhibition (micrococci were more sensitive than *Bacillus* spp. and the latter were more sensitive than Gram negative bacteria) and that an increase in the alkalinity of the medium, which reduced the dissociation of the iron complex, further increased bacterial inhibition. The presence of conalbumin in a medium also enhances pigment production by pseudomonads (Feeney & Nagy, 1952) and suppresses glucose utilization and catalase production by *Staph. aureus* (Schade, 1958), effects which could be produced by a deficiency of available iron.

Using this evidence recent investigators have posed the question: if growth in the egg white is inhibited by the shortage of available iron, can it be overcome by the addition of iron to the white or by a modification of the pH level? Garibaldi (1960), who used a comprehensive collection of common contaminants of rotten eggs, presented data which indicate that either procedure permitted extensive multiplication. Similar results were obtained by Brooks (1960), and Board(1964) demonstrated that extensive bacterial multiplication followed the addition of iron to either the shell membranes or the albumen of intact eggs. Natural or artificial contamination of water with iron salts also results in a high incidence and a fast rate of rotting in washed eggs (Garibaldi & Bayne, 1960; Brant & Starr, 1962; Garibaldi & Bayne, 1962*a*,*b*). This evidence indicates that the chelation of iron by conalbumin is one of the primary factors in preventing the growth of lysozyme insensitive organisms in the albumen.

That this is not the sole factor was shown by Brooks (1960). He was unable to obtain rates of multiplication of pseudomonads in albumen supplemented with iron of the same order as those in a favourable medium. This led him to suggest that an inadequate supply of simple nitrogenous substances might be important, an aspect which was earlier discussed by Haines (1939). The white consists essentially of an aqueous solution of proteins and trace amounts of simpler nitrogenous substances, the latter amounting to *c.* 12 mg/egg (Romanoff & Romanoff, 1949). The albumen contains at least 16 free amino acids the total concentration of which is about 0·15–0·54 μmole/ml in fresh and 2·3 μmole/ml in stored eggs (Ducay, Kline &

Mandeles, 1960). This has often led to the tacit assumption that contaminants of the albumen would be forced to satisfy their nitrogen requirements at the expense of the egg white protein. Such a view did not receive support from the demonstration by Board (1964) that the course of infection of eggs inoculated with non-proteolytic bacteria is essentially the same as that in eggs inoculated with proteolytic organisms.

There is circumstantial evidence that factors other than those discussed above play a role in the antimicrobial defence of the egg. Thus it has been demonstrated, by microbiological assay methods, that there are proteins present in the albumen which make the following unavailable to organisms that require them: biotin (Eakin, Snell & Williams, 1940; Woolley & Longsworth, 1942), riboflavin (Rhodes, Bennett & Feeney, 1959), vitamin B_6 (Evans, Butts & Davidson, 1951). As yet, however, the importance of these factors in the overall defence of the egg has not been demonstrated. Similarly it has been shown *in vitro* but not *in ova* that riboflavin, through its chelation of cations, has a synergistic action with conalbumin in inhibiting microbial growth (Feeney & Nagy, 1952). Ayres (1958) assumed that the antitryptic property of ovomucoid (Balls & Swenson, 1934) is important in the defence of the egg but recent work has shown that it influences neither the growth of organisms (Garibaldi, 1960) nor the proteases produced by them (Masushima, 1958; Board, 1962).

Although storage results in some changes in the egg white proteins, as shown by the loss of clear cut separation by electrophoresis of the different species of protein (Evans & Bandemer, 1946; Stute, 1960; Taylor & Manwell, 1962), there is no evidence that the bactericidal or bacteriostatic properties of the albumen are diminished during short term storage of eggs (Feeney *et al.*, 1952). Such a diminution has been reported for eggs held for 11 months (Sperry, 1913) but this could have resulted from diffusion of iron from the yolk (Schaible *et al.*, 1946) or a reduction in the alkalinity of the albumen (Sharp & Powell, 1931): the properties of the albumen may in fact be ameliorated by metabolic products, e.g. chelating agents, formed during the growth of organisms in the shell membranes (Garibaldi, 1960). Large molecules such as congo red and pyocyanin are known to pass through the shell membranes (Romanoff & Romanoff, 1949; Elliott, 1954) but the influence on the albumen of substances which in practice may penetrate into eggs has not been examined. The assumption has often been made that the production of acids in the microbial utilization of glucose, which amounts to 0·4% of the albumen (Partridge, 1948), causes the pH to be changed to a value suitable for bacterial multiplication. The protein and bicarbonate buffer systems of the albumen are weak in the pH range 7·0–9·0 (Ayres, 1958; Cotterill, Gardner, Cunningham & Funk, 1959) but acid formation does not occur until bacterial multiplication starts in the albumen (Board, 1964; Dr. D. J. Stewart, pers. comm.).

It was noted above that the induction of the secondary phase of multiplication in eggs held at 27° occurs when the yolk makes contact with the shell membranes. The central position of the yolk in a newly laid egg is due primarily to the support it receives from the albuminous sac (Fig. 2). This is a gel in which a transparent phase is permeated by ovomucin fibres (Brooks & Hale, 1959). During storage, the

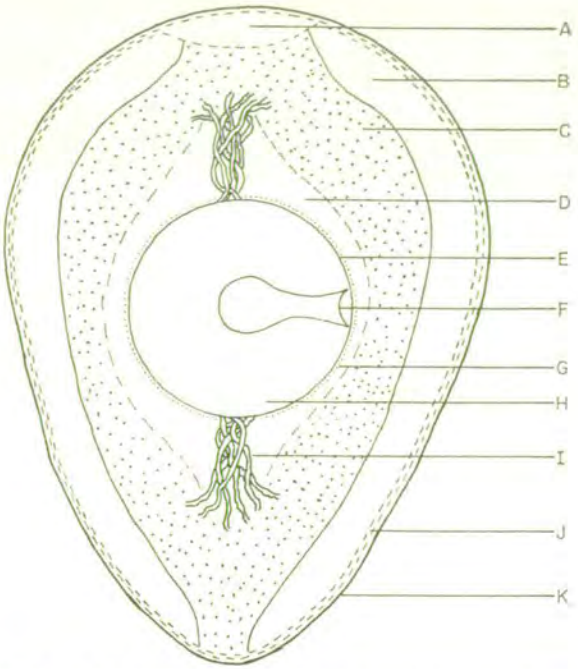

Fig. 2. Structure of the hen's egg as shown by a section through the long axis. A, Air cell; B, outer thin albumen; C, thick albumen (albuminous sac); D, inner thin albumen; E, vitelline membrane; F, blastoderm; G, chalaziferous membrane; H, yolk; I, chalaza; J, shell membranes; K, shell.

rigidity of these decreases rapidly, the albuminous sac shrinks and the freedom of movement of the yolk increases. The physico-chemical basis of these changes has not been established (Brooks & Hale, 1959) but there is evidence of a change in the association of lysozyme and ovomucin (Hawthorne, 1950; Feeney *et al.*, 1952; Cotterill & Winter, 1955; Wilcox, 1955). Under practical conditions it is known that the rate of deterioration of the thick white is influenced by many factors (Sherwood, 1958) of which the initial quality of the albuminous sac, which has a genetic basis (Baker, 1960), and the storage temperature (Jordan, Barr & Wilson, 1954) are of prime importance. There is indirect evidence also that the viscous nature of the albumen may play a part in impeding bacterial migration in the contents of the egg: for example, during the early stages of bacterial growth in the albumen the organisms or pigments produced by them remain localized.

4. Practical Implications

The evidence reviewed to date indicates that bacterial penetration of the shell is of particular importance from the standpoint of controlling rotting in market eggs. This has been accepted by many marketing organizations and they have introduced policies whereby the producer is offered premiums for nest clean eggs. Since it appears to be impracticable to eliminate the soiling of the shell (Brooks, Coles &

Holmes, 1952), producers have been forced to adopt some method of cleaning in order to qualify for the premiums. In the United Kingdom and Australia dry cleaning methods have been advocated because it has been established (Knowles, 1957*a*,*b*) that they result in a low incidence of rotting during storage. This is presumably due to the failure of organisms to penetrate dry shells. Although dry cleaning is desirable from this standpoint, it has not been popular with producers because of the absence until recently of efficient machines capable of cleaning large numbers of eggs. This was possibly the main reason why producers preferred to wash eggs. The influence of this practice on the storage behaviour of eggs has been the subject of much controversy and of many investigations, as witnessed by the reviews of Jenkins & Pennington (1919), Haines (1939), Winter, Burhart & Wettling (1952), Winter, Burhart, Clements & MacDonald (1955), Brooks & Taylor (1955) and Knowles (1956). Although these authors are of the opinion that the incidence of rotting is generally increased by washing, they cite many investigations in which no detrimental effects attended this practice. This is not surprising when it is recalled that microbial penetration of the shell is influenced by many factors. Moreover, in the course of some of the work concerned with the influence of washing on the keeping quality of eggs, it was noted that brown shelled are more resistant than white shelled eggs to rotting (Alford, Holmes, Scott & Vickery, 1950; Trussell, Fulton & Cameron, 1955; Trussell, Triggs & Greer, 1955). This could be taken to suggest that a heritable factor may play a part in the egg's defence but this question has not been examined systematically. It has been shown, also, that the incidence of rotting in washed eggs is greatly increased when the wash water is contaminated with trace amounts of iron (Garibaldi & Bayne, 1960, 1962*a*,*b*). These are obstacles therefore in attempts to predict the storage behaviour of eggs which have been washed under uncontrolled conditions on the premises of the producer. Neither has the addition of disinfectants or antibiotics to the wash water ensured freedom from rotting in stored eggs (Gillespie, Salton & Scott, 1950; Schmidt & Stadelman, 1957; Elliott & Romoser, 1957; Bean & MacLaury, 1959). It would appear, therefore, that success might attend a policy whereby producers send untreated eggs to the packing stations where cleaning could be undertaken.

According to reports appearing in the popular press, egg washing machines have been developed in which the temperature of the wash water is critically controlled, a build up in the contamination of the water is prevented and the content of iron in the water is maintained at a low level. With such machines it would be possible to hold eggs at temperatures of 135·5–158°F. According to Gorseline, Moser & Hayes (1950) it was common practice in Europe and North America in the mid-19th century for farmers to preserve eggs by dipping them momentarily in boiling water. The heat treatment of shell eggs has been the subject of many investigations (Funk, 1943; Romanoff & Romanoff, 1944; Murphy & Sutton, 1947; Funk, 1948; Salton, Scott & Vickery, 1951; Funk, Forward & Lorah, 1954; Scott & Vickery, 1954; Knowles, 1956) and it has been established that 'shell pasteurization' effectively reduces the incidence of rotting in washed eggs and retards the breakdown of the albuminous sac. The latter effect led Funk (1950) to coin the term 'thermostabilization'. Because the incidence of rotting is reduced it has been tacitly

assumed that rot-producing bacteria on the shell and shell membranes are destroyed. Another possible explanation might be that the organisms are not killed but, as a result of physiological damage by the heat treatment, they are unable to grow in the unfavourable environments of the shell membranes. Such a possibility, which is suggested by the investigations concerned with the nutritional requirements of heat treated organisms (e.g. Nelson, 1943; Heater & van der Zant, 1957), would be worthy of further exploration.

The phenomenon of one environmental condition assuming importance when another is approaching the limits of tolerance of an organism might find application in the preservation of eggs. The evidence indicates that the shell membranes, although they are relatively unsuitable for the growth of rot-producing bacteria, do provide a nidus from which infection can spread to the yolk and white. Attention has been given recently to the maintenance of internal quality by storing eggs in sealed cartons in which the CO_2 content and the humidity are controlled (Cotterill & Winter, 1957; Fletcher, Orr, Snyder & Nicholson, 1959; Davis & Beeckler, 1962). It would appear that the behaviour of micro-organisms in the shell membranes of eggs held under these conditions should be examined with the object of determining the conditions of temperature and CO_2 content which prevent microbial multiplication.

In this review no attempt has been made to interpret the antimicrobial defence in terms of its role in protecting the developing embryo. This is due to the lack of a systematic investigation of this aspect, as witnessed by the report of Lutsky & Bell (1953), and ignorance concerning the influence of temperature on the coordination of the defences of the albumen. Investigations in this field could well provide an answer to the question: why does a chicken turn its egg? Is it to prevent a union of the yolk and the shell membranes and a concomitant 'short circuiting' of the antimicrobial defence of the albumen?

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6. References

Alford, L. R., Holmes, N. E., Scott, W. J. & Vickery, J. R. (1950). Studies in the preservation of shell eggs. I. Nature of wastage in Australian export eggs. *Aust. J. appl. Sci.* **1**, 208.

Alderton, G., Ward, W. H. & Fevold, H. L. (1946). Identification of the bacteria-inhibiting, iron-binding protein of egg white as conalbumin. *Archs Biochem.* **11**, 9.

Ayres, J. C. (1958). Methods for depleting glucose from egg albumen before drying. *Fd Technol., Champaign* **12**, 186.

Ayres, J. C. & Taylor, B. (1956). Effect of temperature on microbial proliferation in shell eggs. *Appl. Microbiol.* **4**, 355.

Azari, P. R. & Feeney, R. E. (1958). Resistance of metal complexes of conalbumin and transferrin to proteolysis and to thermal denaturation. *J. biol. Chem.* **232**, 293.

Azari, P. R. & Feeney, R. E. (1961). The resistance of conalbumin and its iron complex to physical and chemical treatments. *Archs Biochem. Biophys.* **92**, 44.

Baker, C. M. A. (1960). The genetic basis of egg quality. *Br. Poult. Sci.* **1**, 3.
Baker, C. M. A. & Manwell, C. (1962). Molecular genetics of avian proteins. 1. The egg white proteins of the domestic fowl. *Br. Poult. Sci.* **3**, 161.
Balls, A. K. & Swenson, T. L. (1934). The antitrypsin of egg white. *J. biol. Chem.* **106**, 409.
Baumgärtner, H. (1957). The effect and occurrence of avidin in raw egg white and dried egg powder. *Ernährungsforschung* **2**, 631.
Bean, K. C. & MacLaury, D. W. (1959). The bacterial contamination of hatching eggs and methods for its control. *Poult. Sci.* **38**, 693.
Bennetts, H. W. (1931). Specific putrefaction of hen eggs due to *Serratia* sp. *Aust. vet. J.* **7**, 27.
Bigland, C. H. & Papas, G. (1953). Experiment in egg penetration by Salmonella. *Can. J. comp. Med.* **17**, 105.
Board, R. G. (1962). *A study of bacterial infection of the hen's egg.* Thesis, University of Edinburgh, Scotland.
Board, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.* **27**, 350.
Board, R. G. (1965*a*). Bacterial growth on and penetration of the shell membranes of the hen's egg. *J. appl. Bact.* **28**, 197.
Board, R. G. (1965*b*). The properties and classification of the predominant bacteria occurring in rotten eggs. *J. appl. Bact.* **28**, 437.
Board, R. G., Ayres, J. C., Kraft, A. A. & Forsythe, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.* **43**, 584.
Board, R. G. & Ayres, J. C. (1965). Influence of temperature on bacterial infection of the hen's egg. *Appl. Microbiol.* **13**, 358.
Board, R. G. & Wilson, E. G. H. (1965). Level of microbial contamination of eggs used for hatching. *Edin. Sch. Agric. Exp. Work* (in press).
Brant, A. W. & Starr, P. B. (1962). Some physical factors related to egg spoilage. *Poult. Sci.* **41**, 1468.
Brooks, J. (1960). Mechanism of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.* **23**, 499.
Brooks, J., Coles, R. & Holmes, N. E. (1952). The problem of dirty eggs. *Agriculture, Lond.* **59**, 311.
Brooks, J. & Hale, H. P. (1959). The mechanical properties of the thick white of the hen's egg. *Biochem. biophys. Acta* **32**, 237.
Brooks, J. & Taylor, D. I. (1955). Eggs and egg products. *Rep. Fd Invest. Bd, Lond.* no. 60. London: H.M.S.O.
Bryant, R. L. & Sharp, P. L. (1934). Effect of washing on keeping quality of hen's egg. *J. agric. Res.* **48**, 62.
Buxton, A. & Gordon, R. F. (1947). The epidemiology and control of *Salmonella thompson* infection of fowls. *J. Hyg., Camb.* **45**, 265.
Cotterill, O. J. & Gardner, F. (1957). Retarding thick white deterioration by holding shell eggs in sealed containers. *Poult. Sci.* **36**, 196.
Cotterill, O. J., Gardner, F., Cunningham, F. E. & Funk, E. M. (1959). Titration curves and turbidity of whole egg white. *Poult. Sci.* **38**, 836.
Cotterill, O. J. & Winter, A. R. (1955). Egg white lysozyme. 3. The effect of pH on the lysozyme-ovomucin interaction. *Poult. Sci.* **34**, 679.
Davis, G. T. & Beeckler, A. F. (1962). Plastic packaging of eggs. 1. Methods of packaging. *Poult. Sci.* **41**, 391.
Ducay, E. D., Kline, L. & Mandeles, S. (1960). Free amino acid content of infertile chicken eggs. *Poult. Sci.* **39**, 831.
Eakin, R. E., Snell, E. E. & Williams, R. J. (1940). A constituent of raw egg white capable of inactivating biotin *in vitro*. *J. biol. Chem.* **136**, 801.
Elliott, R. P. (1954). Spoilage of shell eggs by pseudomonads. *Appl. Microbiol.* **2**, 158.
Elliott, L. E. & Brant, A. W. (1957). Effect of saline and egg shell membrane on bacterial growth. *Fd Res.* **22**, 241.
Elliott, L. E. & Romoser, G. L. (1957). Studies on the recovery of antibiotic residues in egg albumen. *Poult. Sci.* **36**, 365.
Evans, R. I. & Bandemer, S. L. (1956). Egg proteins: Separation of egg white proteins by paper electrophoresis. *J. agric. Fd Chem.* **4**, 802.

Evans, R. I., Butts, H. A. & Davidson, J. A. (1951). The vitamin B6 content of fresh and stored shell eggs. *Poult. Sci.* **30**, 515.

Feeney, R. E., Ducay, E. D., Silva, R. S. & MacDonnell, L. R. (1952). Chemistry of shell egg deterioration: the egg white proteins. *Poult. Sci.* **31**, 639.

Feeney, R. E. & Nagy, D. A. (1952). The antibacterial activity of the egg white protein conalbumin. *J. Bact.* **64**, 629.

Ferdinandov (1944). Quoted from Zagaevsky & Lutikova (1944).

Fleming, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc.* B. **93**, 306.

Fletcher, D. A., Orr, H. L., Snyder, E. S. & Nicholson, A. O. (1959). Effect of oiling, packaging materials and addition of CO_2 on quality of shell eggs held in storage. *Poult. Sci.* **38**, 106.

Florian, M. L. E. & Trussell, P. C. (1957). Bacterial spoilage of eggs. IV. Identification of spoilage organisms. *Fd Technol., Champaign* **11**, 56.

Forsythe, R. H., Ayres, J. C. & Radlo, J. L. (1953). Factors affecting the microbiological population of shell eggs. *Fd Technol., Champaign* **7**, 49.

Fraenkel-Conrat, H. L. & Feeney, R. E. (1950). The metal-binding activity of conalbumin. *Archs. Biochem.* **29**, 101.

Fromm, D. & Margoff, P. H. (1958). The influence of sweating and washing on weight loss, bacterial contamination and interior physical quality of 12-day old shell eggs. *Poult. Sci.* **37**, 1273.

Fromm, D. & Monroe, R. J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg-shell permeability. *Fd Technol., Champaign* **14**, 401.

Friedberger, E. & Hoder, F. (1932). Lysozyme und Flockungsphänomen des Hühnereiklass. *Z. ImmunForsch. exp. Ther.* **74**, 429.

Fuller, R. A. & Briggs, D. R. (1956). Some properties of hen's egg conalbumin. *J. Am. chem. Soc.* **78**, 5253.

Funk, E. M. (1943). Pasteurization of shell eggs. *Res. Bull. Mo. agric. exp. Stn* no. 364.

Funk, E. M. (1948). Experiments on cleaning soiled eggs for storage. *Res. Bull. Mo. agric. exp. Stn* no. 426.

Funk, E. M. (1950). Maintenance of quality in shell eggs by thermostabilization. *Res. Bull. Mo. agric. exp. Stn* no. 467.

Funk, M. E., Forward, I. & Lorah, M. (1954). Maintaining quality in shell eggs by heat treatment. *Res. Bull. Mo. agric. exp. Stn* no. 550.

Garibaldi, J. A. (1960). Factors in egg white which control growth of bacteria. *Fd Res.* **25**, 337.

Garibaldi, J. A. & Bayne, H. G. (1960). The effect of iron on pseudomonas spoilage of experimentally infected eggs. *Poult. Sci.* **39**, 1517.

Garibaldi, J. A. & Bayne, H. G. (1962*a*). Iron and the bacterial spoilage of shell eggs. *J. Fd Sci.* **27**, 57.

Garibaldi, J. A. & Bayne, H. G. (1962*b*). The effect of iron on *Pseudomonas* spoilage of farm washed eggs. *Poult. Sci.* **41**, 850.

Garibaldi, J. A. & Stokes, J. L. (1958). Protective role of shell membranes in bacterial spoilage of eggs. *Fd Res.* **23**, 283.

Gayon, A. (1873). Sur l'alteration spontanée des oeufs. *C.r. Acad. Sci., Paris* **76**, 232.

Gillespie, J. H. & Scott, W. J. (1950). Studies in the preservation of shell eggs. IV. Experiments on the mode of infection by bacteria. *Aust. J. appl. Sci.* **1**, 514.

Gillespie, J. M., Salton, M. R. J. & Scott, W. J. (1950). Studies in the preservation of shell eggs. V. The use of chemical disinfectants in cleaning machines. *Aust. J. appl. Sci.* **1**, 531.

Gordon, R. F. & Tucker, J. F. (1954). Behaviour of *Pseudomonas* sp. and the natural occurrence of the organism in the fowl and its environment. *Contribution* 109 *of* 10*th World's Poult. Congr.* Edinburgh: Department of Agriculture, Scotland.

Gordon, R. F. & Tucker, J. F. (1965). The epizootology of *Salmonella menston* infection of fowls and the effect of feeding poultry food artificially infected with *Salmonella*. *Br. Poult. Sci.* **6**, 251.

Graves, R. C. & MacLaury, D. W. (1962). The effects of temperature, vapour pressure and absolute humidity on bacterial contamination of shell eggs. *Poult. Sci.* **41**, 1219.

Hadley, P. B. & Caldwell, D. W. (1916). The bacterial infection of fresh eggs. *Bull. R.I. agric. exp. Stn* no. 164.

Haines, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of a new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.* **38**, 338.

Haines, R. B. (1939). Microbiology in the preservation of the hen's egg. *Rep. Fd Invest. Bd, Lond.* no. 47. London: H.M.S.O.

Haines, R. B. & Moran, T. (1940). Porosity of, and bacterial invasion through, the shell of the hen's egg. *J. Hyg., Camb.* **40**, 453.

Harry, E. G. (1957). The effect on embryonic and chick mortality of yolk contamination with bacteria from the hen. *Vet. Rec.* **69**, 1433.

Harry, E. G. (1963*a*). Some observations on the bacterial content of the ovary and oviduct of the fowl. *Br. Poult. Sci.* **4**, 63.

Harry, E. G. (1963*b*). The relationship between egg spoilage and the environment of the egg when laid. *Br. Poult. Sci.* **4**, 91.

Hartung, T. E. & Stadelman, W. J. (1962). The influence of metallic ions on the penetration of the egg shell membranes by *Pseudomonas fluorescens*. *Poult. Sci.* **41**, 1590.

Hartung, T. E. & Stadelman, W. J. (1963). *Pseudomonas fluorescens* penetration of egg shell membranes as influenced by shell porosity, age of egg and degree of bacterial challenge. *Poult. Sci.* **42**, 147.

Hawthorne, J. R. (1950). The action of egg white lysozyme on ovomucoid and ovomucin. *Biochim. biophys. Acta* **6**, 28.

Healey, D. J. & Peter, A. M. (1925). The hydrogen ion concentrations and basicity of egg yolk and egg white. *Am. J. Physiol.* **74**, 363.

Heater, C. D. & Zant, W. C. van der (1957). Effects of plating medium on the survival of heat-treated cells of *Pseudomonas fluorescens*. *Fd Res.* **22**, 164.

Horowitz, A. (1903). Vorkommen und Bedeutung der Mikro-organism auf der Äusseren und inneren Körperoberfläche. *J. ber. Fortschr. Lehve pathog. Mikroorg.* **19**, 985.

Jenkins, M. K. & Pennington, M. E. (1919). Commercial preservation of eggs by cold storage. *Bull. U.S. Dep. Agric.* no. 775.

Jordan, R., Barr, A. T. & Wilson, M. L. (1954). Shell eggs—quality and properties as affected by temperature and length of storage. *Bull. Ind. agric. exp. Stn* no. 612.

Knowles, N. R. (1956). The prevention of microbial spoilage in whole shell eggs by heat treatment methods. *J. appl. Bact.* **19**, 293.

Knowles, N. R. (1957*a*). Observations on the keeping quality of dry-cleaned eggs. *Res. exp. Rec. Minist. Agric. Nthn Ire.* **6**, 100.

Knowles, N. R. (1957*b*). The cold storage of dry-cleaned eggs. *Res. exp. Rec. Minist. Agric. Nthn Ire.* **6**, 108.

Korotkova, G. P. (1957). On the protective function of the albuminous membrane in the hen's egg. *Zuhr. Obschahei. Biol.* **18**, 275.

Kraft, A. A., Elliott, L. E. & Brant, A. W. (1958). The shell membrane as a barrier to bacterial penetration of eggs. *Poult. Sci.* **37**, 238.

Kraft, A. A., McNally, E. G. & Brant, A. W. (1958). Shell quality and bacterial infection of shell eggs. *Poult. Sci.* **37**, 638.

Lancaster, J. E. & Crabb, W. E. (1953). Studies on disinfection of eggs and incubators. I. The survival of *Salmonella pullorum*, *thompson* and *typhi-murium* on the surface of the hen's egg. *Br. vet. J.* **109**, 139.

Laschtschenko, P. (1909). Über die keinntotande unt ent wicklungshemmende Wirkung Hünereiweiss. *Z. Hyg. InfektKr.* **64**, 619.

Lifshitz, A. & Baker, R. C. (1964). Some physical properties of the egg shell membranes in relation to their resistance to bacterial penetration. *Poult. Sci.* **43**, 527.

Lifshitz, A., Baker, R. C. & Naylor, H. B. (1964). The relative importance of chicken egg exterior structures in resisting bacterial penetration. *J. Fd Sci.* **29**, 94.

Lineweaver, H. & Hurray, C. W. (1947). Identification of the trypsin inhibitor of egg white with ovomucoid. *J. biol. Chem.* **171**, 565.

Longsworth, L. G., Cannan, R. K. & MacInnes, D. A. (1940). An electrophoretic analysis of egg white. *J. Am. chem. Soc.* **62**, 2580.

Lorenz, F. W., Starr, P. B., Starr, M. P. & Ogasawara, F. X. (1952). The development of *Pseudomonas* spoilage in eggs. I. Penetration through the shell. *Fd Res.* **17**, 351.

Lutsky, I. I. & Bell, A. E. (1953). Antibacterial action of egg albumen and embryonic immunity. *Poult. Sci.* **32**, 285.

Magwood, S. E. (1964). Studies in hatchery sanitation. 3. The effect of air-borne bacterial populations on contamination of egg and embryo surfaces. *Poult. Sci.* **43**, 1567.

MARSHALL, W. & CRUICKSHANK, D. B. (1938). The function of the cuticle in relation to the porosity of eggs. *J. agric. Sci.* **28**, 24.

MASUSHIMA, K. (1958). An undescribed trypsin inhibitor in egg white. *Science, N.Y.* **127**, 1178.

MASSOFF, W. & STOLPMAN, H. J. (1961). Licht und Electromenmikroskopische Untersuchungen und der Schalenhaut und Kalkschale der Huhnereies. *A. Zehlforsch* **55**, 818.

MAY, H. G. (1924). The examination of eggs from infected and immunized hens, with germicidal tests on albumen and blood serum. *Bull. R.I. agric. exp. Stn* no. 197.

MILLER, W. A. & CRAWFORD, L. B. (1953). Some factors influencing bacterial population of eggs. *Poult. Sci.* **32**, 303.

MURPHY, T. W. & SUTTON, W. S. (1947). The pasteurization of shell eggs to prevent storage rot and maintain quality. *Agric. Gaz. N.S.W. Misc. Publ.* no. 3317.

NELSON, F. E. (1943). Factors which influence the growth of heat-treated bacteria. 1. Comparison of four agar media. *J. Bact.* **45**, 395.

OREL, V. (1959). The pseudomonas spoilage of eggs laid by individual hens. *Poult. Sci.* **38**, 8.

OSBORNE, T. B. & CAMPBELL, G. F. (1900). The protein constituents of egg white. *J. Am. chem. Soc.* **22**, 422.

PARASCANDOLE, C. (1893). Cited in Haines (1939).

PARTRIDGE, S. M. (1948). Filter-paper chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238.

PATHAK, R. C., SINGH, C. M. & TANGRI, R. P. (1960). Chick mortality and the contamination of yolks by members of the enterobacteriacae. *Br. vet. J.* **116**, 81.

PLATT, A. E. (1936). Investigations into the nature of the condition known as "floating yolk". *Aust. J. exp. Biol. med. Sci.* **14**, 105.

REID, W. M., MACY, T. A. ,BOYD, F. M., KLECKNER, A. L. & SCHMITTLE, S. C. (1961). Embryo and baby chick mortality and morbidity induced by a strain of *Escherichia coli*. *Poult. Sci.* **40**, 1497.

RETTGER, L. F. (1913). The bacteriology of the hen's egg, with special reference to its freedom from microbic invasion. *Bull. Storrs. agric. Exp. Stn* no. 75.

RETTGER, L. F. & SPERRY, J. A. (1912). The antiseptic and bactericidal properties of egg-white. *J. med. Res.* **26**, 55.

RHODES, M. B., BENNETT, N. & FEENEY, R. E. (1959). The flavoprotein-apoprotein system of egg white. *J. biol. Chem.* **234**, 2054.

RHODES, M. B., BENNETT, N. & FEENEY, R. E. (1960). The trypsin and chymotrypsin inhibitors from avian egg whites. *J. biol. Chem.* **235**, 1686.

RICHARD, O. & MOHLER, A. (1950). Die Mikrobiologie der "Heu-eier". *Mitt. Lebensm. Hyg., Bern.* **41**, 168.

RIEVEL, H. (1939). Die Durchlässigkeit der Eischafe für die Bakerian und ihre Bedeuting für die Kühlhauslangerung. *Proc. 7th World's Poult. Congr.* Cleveland: Waverley Press Inc.

ROMANOFF, A. L. & ROMANOFF, A. J. (1944). A study of preservation of eggs by flash heat treatment. *Fd Res.* **9**, 358.

ROMANOFF, A. L. & ROMANOFF, A. J. (1949). *The Avian Egg.* New York: Wiley & Sons.

ROSSER, F. T. (1942). Preservation of eggs. II. Surface contamination on egg-shell in relation to spoilage. *Can. J. Res.* **20**D, 291.

SALTON, M. R. J. (1957). The properties of lysozyme and its action on micro-organisms. *Bact. Rev.* **21**, 82.

SALTON, M. R. J., SCOTT, W. J. & VICKERY, J. R. (1951). Studies in the preservation of shell eggs. VI. The effect of pasteurization on bacterial rotting. *Aust. J. appl. Sci.* **2**, 205.

SCHADE, A. L. (1958). Siderophillin and conalbumin as physiologically active iron-chelating proteins. *Abstr. Proc. Intern. Congr. Biochem., 4th Congr. Vienna* **127**, 10.

SCHADE, A. L. & CAROLINE, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae, Staphylococcus aureus, Escherichia coli* and *Saccharomyces cerevisiae*. *Science, N.Y.* **100**, 14.

SCHAIBLE, F. J., BANDEMER, S. L. & DAVIDSON, J. A. (1946). Composition of fresh and storage eggs from hens fed cottonseed and non-cottonseed rations. 1. General observations. *Poult. Sci.* **25**, 440.

SCHMIDT, F. J. & STADELMAN, W. J. (1957). Effects of antibiotics and heat treatment of shell eggs on quality after storage. *Poult. Sci.* **36**, 1023.

Scott, W. J. & Vickery, J. R. (1954). Studies in the preservation of eggs. VII. The effect of pasteurization on the maintenance of physical quality. *Aust. J. appl. Sci.* **5**, 89.
Sharp, P. F. & Powell, C. K. (1931). Increase in pH of white and yolk of hen's eggs. *J. indust. engng Chem.* **23**, 196.
Sharp, P. F. & Stewart, G. F. (1936). Effect of relative humidity on the growth of moulds on eggs in storage. *Mem. Cornell agric. exp. Stn* no. 91.
Sharp, P. F. & Whitaker, R. (1927). The relation of the hydrogen ion concentration of egg white to its germicidal action. *J. Bact.* **14**, 17.
Sherwood, D. H. (1958). Factors affecting egg quality—a review. *Poult. Sci.* **37**, 924.
Simkiss, K. (1961). Calcium metabolism and avaian reproduction. *Biol. Rev.* **36**, 321.
Sperry, J. A. (1913). On antiseptic and bactericidal properties of eggs. *Science, N.Y.* **38**, 413.
Stewart, G. F. (1935). The structure of the hen's egg shell. *Poult. Sci.* **14**, 24.
Stokes, J. L. & Osborne, W. W. (1956). Effect of the egg shell membrane on bacteria. *Fd Res.* **21**, 264.
Stokes, J. L., Osborne, W. W. & Bayne, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Fd Res.* **21**, 510.
Stuart, L. S. & McNally, E. H. (1943). Bacteriological studies on the egg shell. *U.S. Egg Poult. Mag.* **49**, 28.
Stute, K. W. (1960). Paper electrophoretic investigations of egg white proteins of chicken eggs. *Arch. Kleintierz.* **24**, 543.
Sullivan, M. X. (1905). Synthetic medium for chromobacterium. *J. med. Res.* **14**, 109.
Taylor, C. M. A. (1960). The genetic basis of egg quality. *Br. Poult. Sci.* **1**, 3.
Taylor, C. M. A. & Manwell, C. (1962). Molecular genetics of avian proteins. 1. The egg white proteins of the domestic fowl. *Br. Poult. Sci.* **3**, 161.
Trussell, P. C., Fulton, C. O. & Cameron, C. I. (1955). Bacterial spoilage of shell eggs. II. Incidence of spoilage in eggs from ninety-four farms. *Fd Technol., Champaign* **9**, 130.
Trussell, P. C., Triggs, R. E. & Greer, B. A. (1955). Bacterial spoilage of shell eggs. III. Farm practices promoting spoilage. *Fd Technol., Champaign* **9**, 134.
Turro, R. (1902). Zur Bakterienverduung. *Zentbl. Bakt. ParasitKde* (Abt 1) **32**, 105.
Tyler, C. (1945). The porosity of egg shells, and the influence of different levels of dietary calcium upon porosity. *J. agric. Sci.* **35**, 168.
Tyler, C. (1953). Studies on egg shells. II. A method for marking and counting pores. *J. Sci. Fd Agric.* **4**, 266.
Tyler, C. (1956). Studies on egg shells. VII. Some aspect of structure as shown by plastic models. *J. Sci. Fd Agric.* **7**, 483.
Tyler, C. (1961*a*). Studies on egg shells. XVI. Variations in shell thickness over different parts of the same shell. *J. Sci. Fd Agric.* **12**, 459.
Tyler, C. (1961*b*). Studies on egg shells. XVII. Variations in membrane thickness and in the true shell nitrogen over different parts of the same shell. *J. Sci. Fd Agric.* **12**, 470.
Walden, C. C., Allen, I. V. F. & Trussell, P. C. (1956). The role of the egg shell and shell membranes in restraining the entry of micro-organisms. *Poult. Sci.* **35**, 1190.
Warner, R. C. & Weber, L. (1951). The preparation of crystalline conalbumin. *J. biol. Chem.* **191**, 173.
Warner, R. C. & Weber, I. (1953). The metal binding properties of conalbumin. *J. Am. chem. Soc.* **75**, 5094.
Weston, W. A. R. D. & Halnan, E. T. (1927). "Black spot" of eggs. *Poult. Sci.* **6**, 251.
Wilcox, F. H. (1955). Evidence of an association between lysozyme level and the quality of egg white. *Poult. Sci.* **34**, 1170.
Williams, J. (1962). Serum proteins and livetins of hen's egg yolk. *Biochem. J.* **83**, 346.
Wilson, J. E. (1945). Infected egg shells are a means of spread of Salmonellosis in chicks and ducklings. *Vet. Rec.* **57**, 411.
Winter, A. R., Burhart, B. & Wettling, C. (1952). Cleaning eggs for market. *Bull. Ohio agric. exp. Stn* no. 710.
Winter, A. R., Burhart, B., Clements, P. & MacDonald, L. (1955). Cleaning eggs with detergents and detergent-sanitizers. *Bull. Ohio agric. exp. Stn* no. 762.
Wolk, J., McNally, E. H. & Spicknall, N. H. (1950). The effect of temperature on bacterial infection of shell eggs. *Fd Technol., Champaign* **4**, 316.
Woolley, D. W. & Longsworth, L. G. (1942). Isolation of an anti-biotin factor from egg white. *J. biol. Chem.* **142**, 285.
Zagaevsky, J. S. & Lutikova, P. O. (1944). Sanitary measures in the egg breaking plant. *U.S. Egg Poult. Mag.* **50**, 17.

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The Microbiology of the Hen's Egg[1]

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I. Introduction

Systematic study of this subject had its genesis in an argument between Donné and Pasteur. The former maintained that vigorous shaking was all that was needed to induce the addling of an egg. Pasteur (see Gayon, 1873) did not always succeed in producing rots by this method but, when successful, he noted large numbers of bacteria in the decomposing yolk and white. He was uncertain of their origin but favored the view that they came from the oviduct. Even when the arguments occasioned by the concept of spontaneous generation had lapsed, research in egg microbiology was dominated by two questions: Why do eggs rot? How, during the distribution of those intended for human consumption, can this be prevented? Perhaps these have been the main cause of the *ad hoc* nature of the research and for the piecemeal growth of our knowledge—witness, also, this essay in which strings of references have had to be given because of the absence of incisive studies.

Moreover the strong applied bias may be a reason why the data have had such a limited impact in general biology. Thus one can perceive a widening of the gulf between pure and applied research and a loss of rapport between disciplines that have an interest in the problem. This is to be regretted since those engaged in molecular biology, for example, might find that the whole egg could

[1] Dedicated to Dr. T. Gibson on the occasion of his seventieth birthday.

provide a model system for testing the biological implications of hypotheses based on detailed study of one of its components. Similarly, such information could lead egg technologists to a fuller appreciation of the antimicrobial defense of the egg and, perhaps, suggest ways in which the biological properties of a component could be exploited or its action aped in the food and allied industries. Likewise, one would imagine that the fate of the developing embryo would be of as much concern to the zoologist as to persons employed in the hatchery industry, yet this surmise receives little support from the literature (Beer, 1967; Lack, 1968). Such considerations influenced the organization of this essay and an attempt has been made to place the data in a biological rather than an applied setting.

II. The Egg

A. Structure

The egg (Fig. 1) is a complex physicochemical system in which enzyme-mediated energy transfer and chemical transformations are limited, in the main, to the cells of the blastoderm (Brooks and Taylor, 1955; Shenstone, 1968). With eggs intended for human consumption, their activities are minimized by low-temperature storage. This delays also the rate of deterioration of the physicochemical systems and loss of the structural integrity of the main components of an egg. The principal changes occurring in a stored egg are summarized in Table I. Of these, the breakdown of the albuminous sac (Brooks and Hale, 1959) and the stretching and weakening of the vitelline membrane (Fromm, 1967) are, from a commercial viewpoint, the chief causes of the loss of "quality" (Wells, 1968) and prolonged storage is required before there are demonstrable changes in the chemical composition of eggs (Evans *et al.*, 1950). During the formation of an egg, a genetic code along with the materials (Table II) needed for its interpretation to the chick stage are included within the shell and its two membranes (Fig. 1).

Intimate association of the hen and eggs ends at oviposition—there is nothing comparable to the uterine immunity discussed by Brambell (1958). The prenatal stages of development occur in an environment separated from that of the parent by the shell. In many ways the egg can be viewed as an ecosystem with a need only for exchange of respiratory gases, a source of heat, and regular movement. This stage of development has been studied in great detail, as witnessed by the monograph of Romanoff (1967), and when the bioenergetics are

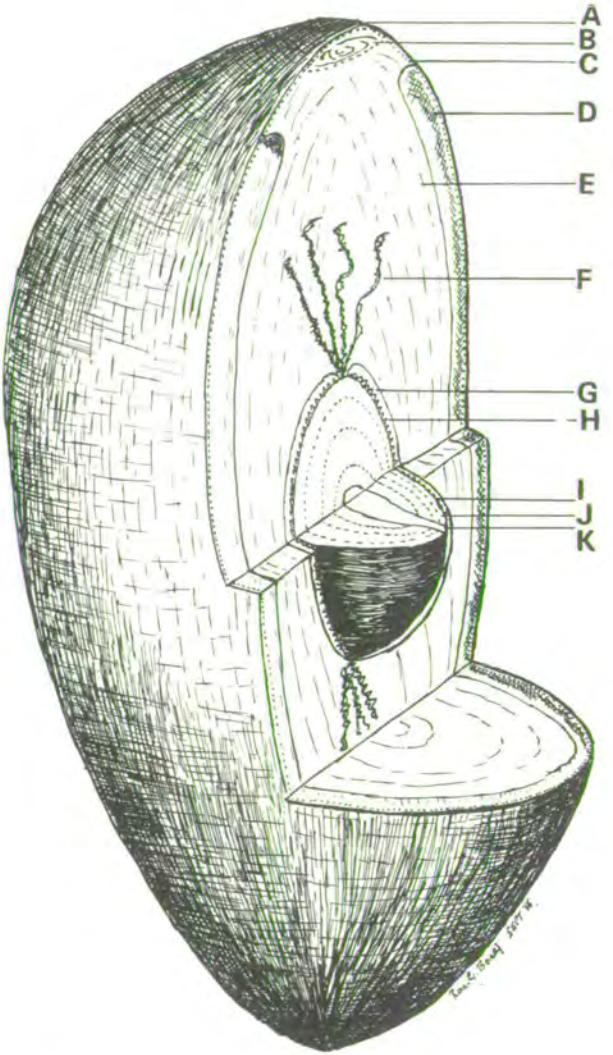

FIG. 1. An artist's impression of the hen's egg as shown by cutaway sections. A, Shell; B, air cell; C, inner shell membranes; D, outer thin white; E, albuminous sac; F, chalazae; G, inner thin white; H, chalaziferous membrane; I, vitelline membrane; J, yolk; K, latebra.

represented graphically, it is found not surprising that the pattern is similar to that which summarizes the changes occurring in a pure culture of a bacterium (Dean and Hinshelwood, 1966). There would

TABLE I
CHANGES OCCURRING IN STORED EGGS

Change	Cause	Effect	Environmental factors influencing	References
1. Shrinkage of cuticle	(*i*) Shrinkage of the vesicles (*ii*) Diminution of inter-vesicular air space	Increased porosity of shell?	In egg cleaning, temperature of water, type of detergent	Simons and Wiertz (1966)
2. Formation of air cell	Differential contraction of the shell and egg contents	Inner shell membrane pulled away from the outer shell membrane	Ambient temperature	Romanoff and Romanoff (1949)
3. Alkaline drift in in albumen	Diffusion of CO_2	Breakdown of the buffer system of albumen	Temperature and composition of atmosphere of store	Cotterill *et al.* (1958)
4. Increase in density of albumen	(*i*) Evaporation	Increase in volume of air cell	Temperature, relative humidity, shell thickness	Wells (1968)
	(*ii*) Absorption of water by yolk	Decrease in viscosity of yolk Stretching and weakening of vitelline membrane	Temperature and differences in osmotic pressure between yolk and white	Feeney *et al.* (1956) Smith (1934)

5. Deterioration of albuminous sac	Unknown in detail: interaction of lysozyme and ovomucin considered important (Cotterill and Winter 1954a,b, 1955)	(*i*) Fracture of fibers connecting the sac to the shell membranes at the poles of the egg (*ii*) Contraction of albuminous sac around yolk (*iii*) Movement of inner thin white to outside of sac (*iv*) Contraction of the chalazae (*v*) Loss of gelation	(a) Temperature (b) Heritable factors (c) CO_2 content of storage atmosphere (d) Treatment of egg prior to storage—viz., protection by thermostabilization (pasteurization)	Brooks and Hale (1959) Baker and Stadelman (1958) Baker (1960) Rutherford and Murray (1963) Murray and Rutherford (1963) Funk (1943)
6. Increased buoyancy of yolk	Combination of 4 (*i*) and (*ii*)	Yolk moves away from center and comes to rest against shell membranes due to 5 (*i*), (*ii*), (*iv*), and (*v*)	5a, b, c, and d	Romanoff and Romanoff (1949)

TABLE II

COMPOSITION OF THE HEN'S EGG[a]

Distribution of Egg Contents		
	Amount (%)	Grams
Total	100.0	51.6
Water	73.6	38.0
Solids	26.4	13.6
Organic matter	25.6	13.2
Proteins	12.8	6.6
Lipids	11.8	6.1
Carbohydrates	1.0	0.5
Inorganic matter	0.8	0.4

Composition of Albumen	
	Amount (%)
Total	100.0
Water	87.9
Solids	12.2
Organic matter	11.6
Proteins	10.6
Lipids	Trace
Carbohydrates	0.9
Inorganic matter	0.6

Protein Composition of Albumen	
Component	Percent (approx.) of egg white solids
Ovalbumin	54
Conalbumin	13
Ovomucoid	11
Lysozyme	3.5
Ovomycin	1.5
Flavoprotein-apoprotein	0.8
Ovoinhibitor	0.1
Avidin	0.05
Unidentified proteins (mainly globulins)	8

Composition of Egg Shell	
	Amount (%)
Total	100.0
Water	1.6
Solids	98.4
Organic matter	3.3
Proteins	3.3
Lipids	0.03
Inorganic matter	95.1

Composition of Yolk	
	Amount (%)
Total	100.0
Water	48.
Solids	5.1
Organic matter	5.0
Proteins	16.6
Lipids	32.6
Carbohydrates	1.0
Inorganic matter	1.1

[a]Compiled from data given by Romanoff and Romanoff (1949); Brooks and Taylor (1955); Parkinson (1966); Shenstone (1968).

appear to be, therefore, an *a priori* need for such a limited universe as an egg to be endowed with means whereby the embryonic cells are (*i*) protected from physical damage due to sudden and violent changes in the surrounding environment and (*ii*) maintained as a "pure culture." When the relevant facts are assembled, this thesis can be supported.

B. Strength

When considering protection from physical damage, various roles can be proposed for the structural components of the hen's egg (Table III). Of these, the shell provides an example of a structure whereby natural selection seems to have favored a compromise. It has to provide the prebrooding and prenatal stages with protection against damage by impact or crushing as well as allowing the exchange of respiratory gases (6.6 gm., O_2; 7.6 gm., CO_2/21 days; Romanoff, 1967). To allow this exchange the shell contains 7.0–17 $\times$ 10^3 pores (Tyler, 1953), the diameters of which are in the range 9–35 μ (Romanoff and Romanoff, 1949; Tyler, 1956). When examining their distribution, Tyler (1955) noted that the arrangement lay somewhere be-

TABLE III

The Role of Components of the Egg in the Defense of the Embryo[a]

Component	Role
1. (a) Cuticle	Barrier to microbial invasion (a, b, and c)
(b) Shell	Control of rate of evaporation (a, b, and c)
(c) Shell membranes	Regulation of gaseous exchange (a and b)
	Protection against crushing (b and c)
2. Air cell	Control of pressure within egg
3. Albumen	Cushioning against damage due to sudden movement of the egg
	Reservoir of water
	Lag against violent fluctuations in temperature
	Impediment to microbial movement
	Control of rate and extent of microbial growth
	Passive immunity in young chick?
4. Chalaziferous and vitelline membranes	Physical, and to a less extent, chemical isolation of the yolk from the white
5. Yolk	Exchange of material between the yolk and white is controlled by the diffusion gradient existing beneath the vitelline membrane

[a] The roles of the components listed above have been postulated from data given in Romanoff and Romanoff (1949).

tween randomness and complete uniformity. He believed that this spacing maintained a balance between the need for gaseous exchange and the requirement for the shell to resist fracture or crushing. These latter attributes are also of commercial importance (Baskett *et al.*, 1937; Brooks and Hale, 1959; Hale, 1950; Brooks, 1960a; Simons *et al.*, 1966) but, in spite of our detailed knowledge of the structure (Simons and Wiertz, 1963) and chemical composition (Simkiss, 1961, 1968), the actual basis of the shell's strength awaits definition (Tyler, 1968).

C. Defense

What manufacturer would be so foolhardy as to attempt to pack a perishable commodity (Table II) in a semipermeable membrane within a fragile, porous shell and have it distributed and marketed under uncontrolled conditions? Yet this is what the hen appears to have done for many thousands of years. It is thus reasonable to assume that, through selection, some form of defense has evolved whereby the blastoderm and its supply of nutrients are protected from microbial attack during the prebrooding period and, whereby, the early stages of embryo development occur without interference from microbes be they bacteria, fungi, protozoa, worms, or maggots. When discussing this topic Tokin (1959) implied that immunological properties of the egg, particularly of the albumen, provides a passive immunity. Yet, to all intents and purposes, the albumen is free from antibodies. This is not altogether surprising since, as will be seen later (Table VII), the albumen is liable to invasion by organisms with which the hen is unlikely to have had intimate or recent contact. Consequently its chances of containing sufficient antibodies to repel a specific organism are remote. Antibodies are included in the yolk (Frazer *et al.*, 1934; Brandley *et al.*, 1946), and the titer reflects the concentration in the plasma of the hen (Patterson *et al.*, 1962). As these are absorbed from the yolk by the young chick, they could provide a means—a passive immunity—whereby the latter is afforded protection against pathogens that have recently afflicted the mother and are thus liable to be present on or in the hen. Phagocytic cells, even if they did occur in eggs, would seem to be incapable of providing an efficient defense since there would be no method by which they could be transported to the site of infection and, once there, they would lack the complementary action of antibodies.

As the two classic forms of immunity do not and, possibly, could not function efficiently, another means of defense has to be sought. The

early studies of the hen's egg indicated that the albumen kills or prevents the growth of a wide range of microorganisms, whereas, the yolk or a mixture of yolk and white does not (for references, see Haines, 1939). This has been confirmed in recent years (Brown *et al.*, 1966a; Zagaevsky and Lutikova, 1944; Ayres and Taylor, 1956). This implies that the structural integrity of the yolk and white are important in the egg's defense. Likewise it has been demonstrated that the fracturing of the shell increases an egg's susceptibility to microbial degradation (Brown *et al.*, 1966c). In the past 20 years, intensive study of the properties of the proteins of the albumen has allowed a tentative but as yet incomplete description of the means whereby the young embryo is maintained as a "pure culture." This work has been summarized in Table IV, and it will be noted that the albumen can be viewed as a medium that is unsuitable for microbial growth. Of the factors listed, lysozyme, conalbumin, and the alkaline reaction appear to be of primary importance.

1. Lysozyme

In 1909, Laschtschenko noted that *Bacillus subtilis* was lyzed when added to egg white; however, this did not occur when the white had been held at 65°–70°C. for 30 minutes. The enzymatic nature of this phenomenon was recognized by Fleming (1922) and he gave the name lysozyme to the lytic agent. Today this enzyme is considered to be a *N*-acetylhexosaminidase and, along with related enzymes from other sources, it is classified as a muramidase (Salton, 1964). The lysozyme of egg white has been studied to the extent that the amino acid sequence of the protein is now known and, that, from the data concerning the configuration of the macromolecule, it is possible to interpret its catalytic activity in stereochemical terms (Symposium, 1967). In the past 20 years also, this enzyme has been extensively used in studies concerned with the chemical composition and organization of the bacterial cell wall (Salton, 1964; Weidel and Pelzer, 1964; Martin, 1963; Rogers, 1965; Rogers and Perkins, 1968).

The cytoplasmic membrane of the eubacterial cell is contained within a rigid macromolecular net, the basal structure (Work, 1957), murein layer (Martin, 1963), or murein sacculus (Weidal and Pelzer, 1964). In some ways, the structure can be considered to have a role similar to that of the egg shell. It gives the cell a characteristic shape and protection against damage that could ensue from sudden changes in osmotic pressure. The net is formed from a heteropolymer, trivially referred to as mucopeptide (Mandelstam and Rogers, 1959), glyco-

TABLE IV
POSTULATED COMPONENTS OF THE ANTIMICROBIAL DEFENSE OF THE HEN'S EGG

Component	Action	Contribution to defense[a]	References
1. Lysozyme	Lysis of cell walls of eubacteria Flocculation of bacterial cells Hydrolysis of β1–4 glycosidic bonds	A	Laschtschenko (1909); Fleming (1922); Friedberger and Hoder (1932); Berger and Weiser (1957)
2. Conalbumin (interaction with 5; possible interaction with 3)	Chelation of iron, copper, and zinc	A	Schade and Caroline (1944); Alderton *et al.* (1946)
3. Riboflavin (possible interaction with 2)	Chelation of cations	B	Feeney and Nagy (1952); Ramsey and and Wilson (1957)
4. Glucose (possible interaction with 2 and 3)	Repression of respiratory capacity of facultative anaerobes?	B	Schade *et al.* (1968)
5. pH 9.6 (interaction with 2)	Maximizes chelating potential of conalbumin Provides an unsuitable environment for many organisms	A	Schade and Caroline (1944); Garibaldi (1960)

6. Low content of nonprotein nitrogen (possible interaction with 2, 4, and 5)	Elective for nutritionally nonfastidious organisms	C	Haines (1939); Ducay *et al.* (1960)
7. Avidin	Combination with biotin	B	Eakin *et al.* (1940); Wolley and Longsworth (1942)
8. Apoprotein	Combination with riboflavin	B	Rhodes *et al.* (1959)
9. Ovoinhibitor	Inhibition of fungal proteases	C	Matsushima (1958)
10. Ovomucoid	Inhibition of trypsin	C	Delezenne and Pozerski (1903); Feeney *et al.* (1963)
11. Uncharacterized proteins			
A	Inhibition of trypsin and chemotrypsin	C	Rhodes *et al.* (1960)
B	Combination with vitamin B_6	C	Evans *et al.* (1951)
C	Chelation of calcium	C	Abels (1936)
D	Inhibition of ficin and papain	C	Fossum and Whitaker (1968)

[a]Role has been demonstrated in intact eggs (A) or with albumen *in vitro* (B) or postulated on basis of information in literature (C).

peptide (Strominger, 1962), glycosaminopeptide (Salton, 1964) etc., which consists of an axial filament formed from *N*-acetylglucosamine and *N*-acetylmuramic acid (linkage β1-4 and β1-6 glycosidic bonds). Side chains consist of peptides formed from some or all of the following: glutamic acid, alanine, lysine, 2,6-diaminopimelic acid, and glycine (Rogers and Perkins, 1968). The β1-4 bonds are attacked by lysozyme (Berger and Weiser, 1957; Salton and Ghuysen, 1960).

In his studies, Fleming (1922) selected the organism *Micrococcus lysodeikticus*, because of its great sensitivity to lysozyme. It is noteworthy that, on a dry weight basis, the heteropolymer contributes upward of 90% of the cell wall of this organism (Perkins and Rogers, 1959). In consequence the marked sensitivity to lysozyme is associated with the exposed position of many of the β1-4 glycosidic bonds. A similar situation is found in *Bacillus megaterium;* depolymerization of the cell wall causes this rod shaped organism to assume a spherical form (a protoplast) that is quickly lyzed unless suspended in an isotonic solution (Weibull, 1953a,b). The organisms noted to date give a positive reaction in Gram's staining method. Although the cell walls of such organisms are rich in the heteropolymer, they show varying resistance to lysozyme. In some instances, as with *Listeria monocytogenes*, resistance appears to be associated with lipids masking the murein layer (Ghosh and Murray, 1967), while with others (e.g., resistant strains of *M. lysodeikticus* and naturally occurring strains of *Staphylococcus aureus*) the substitution of *N*-acetyl by *O*-acetyl (Brumfitt, 1959; Park and Griffith, 1964) or *N*-propionyl residues (Hara and Matsushima, 1967) prevent lysozyme from making effective contact with its substrate.

The murein sacculus makes a relatively small contribution (as low as 5–10%) to the cell walls of gram-negative bacteria (Rogers and Perkins, 1968) and it is buried beneath laminae of lipoproteins and lipopolysaccharides (Kellenberger and Ryter, 1958; Burge and Draper, 1967a,b,c). How these layers are joined is not known, but ionic and hydrogen bonding have been suggested (Gray and Wilkinson, 1965a,b; Rogers, 1965; Wolin, 1966; Birdsell and Cota-Robles, 1967; Asbell and Eagon, 1966; Nermut and Murray, 1967; Weinbaum *et al.*, 1967; Cox and Asbell, 1968). With the possible exception of very young cells (Birdsell and Cota-Robles, 1967), the outer layers of the cell wall provides a permeability barrier whereby lysozyme is prevented from reaching its substrate. Sensitivity to the enzyme can be imposed by treatments that disrupt the surface architecture of the cell wall. This

can be achieved by incubation under alkaline conditions (Zinder and Arndt, 1956; Vos, 1964; Birdsell and Cota-Robles, 1967), freezing and thawing (Kohn, 1960), or treatment with alkaline solutions of ethylenediaminetetraacetic acid (Repaske, 1956, 1958; Vos, 1967). Lysis expresses itself by a decline in the opacity of a suspension of treated cells, and this can occur without the prior formation of the spherical (spheroplast) form. Thus, Vos (1964) and Asbell and Eagon (1966) have noted that treated cells retain their normal morphology even though they are extremely sensitive to slight changes in osmotic pressure. This suggests (Carson and Eagon, 1966; Weinbaum and Markham, 1966) that the outer layers contribute to the overall strength of the cell wall of gram-negative bacteria and that this fact will have to be taken into account when the action of lysozyme within the egg is being examined.

2. *Conalbumin (Ovotransferrin)*

This protein was first isolated from eggs by Osborne and Cambell (1900). It is a glycoprotein with properties similar to transferrin (siderophilin), the β_1-globulin of serum (Marshall and Deutsch, 1951; Karminiski and Durieux, 1956; Aisen *et al.*, 1966); their chief difference is in their sialic acid content of the carbohydrate moiety (Williams, 1962). The proteins have molecular weights in the range 70–90 $\times$ 10^3 (Laurell and Ingleman, 1947; Bain and Deutsch, 1948; Warner and Weber, 1951) and they act as chelating agents (Alderton *et al.*, 1946; Fraenkel-Conrat and Feeney, 1950; Schade *et al.*, 1949). The binding of iron, copper, and zinc is through ionic bonds and binding constants are high; the estimates for the pK_1 and pK_2 at pH 7.4 being 27.7 and 30.3, respectively (Davis *et al.*, 1962). The iron within the complex is trivalent (Ehrenberg and Laurell, 1955) as is that occurring in eggs (Halkett *et al.*, 1958). The conalbumin-iron complex is more resistant, than is conalbumin alone, to enzymic digestion. thermal denaturation etc. (Fuller and Briggs, 1956; Azari and Feeney, 1958, 1961). This feature has been exploited in methods devised for the isolation and purification of the protein (Azari and Baugh, 1967), the sterilization of plasma (Keller and Pennell, 1958), and in a method for the pasteurization of egg albumen (Cunningham and Lineweaver, 1965).

In the majority of commercial strains of laying hens, the transferrin locus may be homozygous (Tf_a/Tf_a) or heterozygous (Tf_a/Tf_b) whereas the wild type is apparently homozygous for the allele Tf_b (Morton

et al., 1965). Such differences at the chromosome level are reflected in the rate of migration and homogeneity or otherwise of the conalbumin "band" during electrophoresis (Lush, 1961; Ogden *et al.*, 1961; Baker and Manwell, 1962; Baker, 1968). The liver appears to be the most likely site of transferrin synthesis and it has been demonstrated that amino acids can be included in conalbumin by cells and cell debris derived from the oviduct (Mandeles and Ducay, 1961; Williams, 1962; Carey, 1966).

Varieties of conalbumin are widely distributed in the eggs of different species of birds (Clark *et al.*, 1963; Baker, 1968). In the domestic hen it accounts for 10% of the total egg white solids (Longsworth *et al.*, 1940; Alderton *et al.*, 1946); it is uniformly distributed throughout the white (Feeney *et al.*, 1952) and its sequestering power is not detectably reduced by short-term storage (Feeney *et al.*, 1952) unless iron migration from the yolk (Schaible *et al.*, 1946) is expedited through the diffusion gradient at the periphery of the yolk, being modified by physical (Hale, 1950) or chemical methods (Phelps *et al.*, 1965). The bacteriostatic action of conalbumin and its dependance upon a relatively low hydrogen-ion concentration was noted by Schade and Caroline (1944) and confirmed by Brooks (1960b) and Garibaldi (1960). When pure preparations of conalbumin or transferrin are used to inhibit microbial growth, it has been established that they must be present in stoichiometric excess of the iron found in a medium by chemical analysis (Fraenkel-Conrat and Feeney, 1950; Feeney and Nagy, 1952). Inhibition expresses itself by an increase in the lag phase of growth and a decreased rate of multiplication once growth has begun (Theodore and Schade, 1965a), the actual rate and extent of growth being a function of the percentage iron–saturation of the ligand (Schade, 1958, 1963). Feeney and his collaborators have noted that different organisms show different degrees of inhibition (micrococci are more sensitive than *Bacillus* spp. and the latter are more sensitive than gram-negative bacteria).

Indirect evidence that bacteriostasis stems from a disturbed iron metabolism came with the observations (Feeney and Nagy, 1952; Garibaldi, 1967) that conalbumin enhances the production of pigment by pseudomonads. This is a typical response of these organisms when growing in media having a reduced iron content (King *et al.*, 1948; Totter and Moseley, 1953; Paton, 1959). As pseudomonads and physiologically related organisms depend on a cytochrome-containing electron transport system, it would be of interest to know how this system operates when the organisms are exposed to conalbumin. It

has been suggested that the organisms scavenge the free Fe^{3+} that is always present through dissociation of the complex (Fraenkel-Conrat and Feeney, 1950; Feeney, 1951; Feeney and Nagy, 1952), or that they themselves form a chelate (Garibaldi and Neilands, 1956; Garibaldi, 1960) which, through competitition, causes iron to be released from the conalbumin complex in much the same way as is brought about by 8-hydroxyquinoline or citric acid (Feeney and Nagy, 1952).

A more detailed picture of the bacteriostatic action of conalbumin has come from studies concerned with the growth and metabolic activities of *Staphylococcus aureus*. With media containing known amounts of iron, it has been shown that the initiation, rate, and extent of growth are related directly to the concentration of ionic iron (Theodore and Schade, 1965a) or, when the medium contains either conalbumin or transferrin, that the growth rate is a function of the percentage of iron-saturation of the ligand (Schade, 1960, 1963; Theodore and Schade, 1965a). Cells harvested from a carbohydrate-free medium containing optimal amounts of iron are capable of complete oxidation of glucose, pyruvate, acetate, formate, and Krebs cycle intermediates (Theodore and Schade, 1965b; Schade *et al.*, 1968). These findings are in accord with those of Strasters and Winkler (1963), who demonstrated that *S. aureus* has a pentose and a TCA cycle; the latter is of primary importance when the organisms grow aerobically in nutrient broth. The presence of glucose in a medium containing readily available iron represses the synthesis of catalase and reduces the cells' capacity to oxidize the substrates noted previously (Theodore and Schade, 1965b); at the same time it enhances the production of dehydrogenases for glyceraldehyde 3-phosphate and lactic acid (Strasters and Winkler, 1963). Moreover, the exclusion of molecular oxygen from cells growing in a complex medium results in a reduction in the synthesis of cytochromes (Strasters and Winkler, 1963; Jacobs and Conti, 1965; Conti *et al.*, 1968), and there are reasons to believe that the concentration of these substances play a role in controlling the synthesis of lactate dehydrogenase (Garrard and Lascelles, 1968). An analogous repression of oxidative capacity follows the addition of glucose to a culture of *Escherichia coli* (Gray *et al.*, 1966a,b). It is noteworthy that Theodore and Schade (1965b) caused modifications of this type by deliberately minimizing the concentration of iron in a carbohydrate-free medium. They found, moreover, that the presence of glucose in such a medium further reduced the oxidative capacity of the organisms. Although such cells must rely upon glycolysis, they appear to differ from anaerobically grown cells in that the end products

of fermentation include pyruvate, acetate, and acetoin whereas, normally, lactic acid predominates (Gardner and Lascelles, 1962; Collins and Lascelles, 1962). Thus the picture emerging from the studies of Schade and his collaborators indicates that inadequate levels of iron causes a facultative anaerobe such as *S. aureus* to change from respiration to glycolysis, and, under the most exaggerated conditions, this could be expected to reflect itself in a reduced amount of growth since the latter is a relatively inefficient method for ATP synthesis (Bauchop and Elsden, 1960). To date, however, the data do not permit a complete interpretation and this will not be possible until more is known concerning the control mechanisms in microorganisms. Moreover it would be of interest to know, in view of the low levels (0.14–0.54 μmole/ml.) of amino acid in the albumen of the egg (Ducay *et al.*, 1960), whether or not organisms become nutritionally more demanding if iron starvation were to impair the efficiency of an amphibolic system (Davis, 1961), such as the TCA cycle.

As the white is primarily a reservoir of water during early embryo development (Romanoff and Romanoff, 1949) its other components appear to be concerned, along with the shell, in isolating the major food reserve and the young cells from microorganisms on the mother or in the nest. In other words, they contribute to the isolation of the ecosystem in which early prenatal development occurs. Later they are absorbed by the embryo (Baker, 1968)—does this result in an enhancement of the passive immunity of the young chick? Of the components listed in Table IV, available evidence restricts serious discussion to lysozyme and conalbumin. It is noteworthy that the former is widely distributed in the tissues and secretions of the animal body (Fleming, 1922) and that it is a normal constituent of the phagocytic cell (Hirsch, 1965). Recent studies have suggested that conalbumin and related substances may be an important component of the antimicrobial defense of man and animals (Summers and Hasenclever, 1964; Sword, 1966; Caroline *et al.*, 1964). It is interesting to note that the egg appears to be endowed with those components of the animals' antimicrobial defense system which do not require a vascular system, nervous or hormonal control, and which can function over a range of temperatures. Moreover, do these components constitute the earliest form of immunity in animals? This query is posed because of the claims that lysozyme may be a primitive enzyme (Manwell, 1967) and because of the observation that conalbumin-like substances are plentiful in body fluids of animals in which it is difficult to elicit antibody production (Good *et al.*, 1967; Manwell, 1963).

III. The Course of Infection

The majority of early investigations on the rotting process tend to be muddled since the workers failed to appreciate that addling was merely the culmination of a sequence of events. This was recognized by Gillespie and Scott (1950). They defined three phases: (1) infection and microbial penetration of the shell, (2) colonization of the shell membranes, and (3) contamination of the albumen. This concept still provides a valid approach in applied research, but it does suffer from the fact that emphasis is placed on a particular stage, which detracts from an overall view of the process. A desirable level of integration can be achieved when the egg is considered as an ecosystem and the commercially important stages noted above are considered within a framework provided by the concepts of ecology. Thus the term *association* can be used for the characteristic flora of rotten eggs and its genesis under practical conditions can be considered to be determined by (1) the *infection* of the shell and shell membranes, (2) conditions obtaining in the egg (*intrinsic* factors), (3) conditions external to the egg (*extrinsic* factors), and (4) the properties of the organisms making up the association (*implicit* factors).

A. Infection

Previous reviewers (Brooks and Taylor, 1955; Board, 1968) have deduced that microorganisms are absent from the majority of eggs laid by healthy hens. Even when contamination does occur, the types of organisms in the egg at oviposition differ markedly from those in rotten eggs or those which fail to hatch (Harry, 1963a). Moreover their presence in the yolk sac at the time of hatching is of no practical importance since it appears that they would be unlikely to become established in the young chick (Fuller and Jayne-Williams, 1968). The shell can be infected when passing through the vent (Stuart and McNally, 1943) but the chief contamination results from the shell's contact with dirty surfaces (Rosser, 1942; Board *et al.*, 1964; Harry, 1963b). The extent of contamination is indicated by the data given in Table V. As would be expected, the shell harbors a heterogeneous population (Table VI) in which gram-positive organisms are dominant—does this reflect their resistance to drying? When the major components of the flora are considered, it seems reasonable to deduce that dust, soil, and feces are the major depots of the common contaminants. Infection is largely confined to the surface of the shell of nest clean eggs (Dr. K. Büchli, personal communication), and less than

TABLE V

LEVELS OF MICROBIAL CONTAMINATION OF THE SHELL OF THE HEN'S EGG

Source of supply	No. eggs examined	Number of micro-organisms/shell		Grade and/or treatment of eggs	Reported by
		Mean	Range		
Farm	36	9.5×10^3	–[a]	Handled with gloved hands	Rosser (1942)
Batteries	25	2.2×10^4	2.5×10^3–8.1×10^4	–	Harry (1963b)
Experimental farm	–[a]	6.3×10^4	1.0×10^6–1.0×10^6	–	Forsythe *et al.* (1953)
Packing station	72	7.0×10^4	–	–	Rosser (1942)
Farm	36	1.5×10^5	–	–	Rosser (1942)
Shops and farms	130	1.3×10^5	–	–	Haines (1938)
Packing station	–	2.2×10^5	3.0×10^2–1.0×10^7	Clean, Grade A	Board *et al.* (1964)
Deep litter	25	3.5×10^5	6.2×10^3–2.4×10^6	–	Harry (1963b)
Packing station	77	9.7×10^5	1.0×10^3–1.9×10^7	Lightly soiled, Grade B	Board *et al.* (1964)
Deep litter	96	3.1×10^6	5.0×10^2–1.0×10^7	Clean and lightly soiled	Board and Wilson (1965)

[a] –, Details not given in original report.

1% of such eggs rot during storage (Brooks and Taylor, 1955). The organisms on the shell do not multiply unless eggs are held under humid conditions (Sharp and Stewart, 1936; Haines, 1938; Forsythe *et al.*, 1953; Board *et al.*, 1964); with pathogens, such as salmonellae, death can occur (Mellor and Banwart, 1965) unless the eggs are held under moist, chilled conditions or the organisms are included in dried mud or fecal material (Wolk *et al.*, 1950; Lancaster and Crabb, 1953; Cotterill and Gardner, 1957; Magwood, 1964b; Rizk *et al.*, 1966b). Thus under normal conditions the shell is contaminated at its surface with organisms which tend to remain quiescent.

When the composition of the flora of the shell (Table VI) is compared with that of rotten eggs (Table VII), it is notable that the incidence of gram-positive organisms in the former is contrary to that in the latter. Thus, potential rot-producing bacteria make only a small contribution to the initial contamination of the shell, even though such organisms (e.g., *Pseudomonas*) may be widely disseminated in the hen's environment (Gordon and Tucker, 1954). It would seem, therefore, that modern methods of poultry husbandry do not impose conditions elective for gram-negative organisms. This situation does not obtain in the hatchery where undesirable microorganisms can be selected unless the hatchery is designed so that cross-contamination is minimized and a high level of hygiene maintained (Magwood, 1964a,b; Magwood and Marr, 1964; Nichols *et al.*, 1967).

B. Penetration

It has long been recognized that the shell imposes a barrier to microbial invasion of the egg (Haines, 1939; Williams and Whittemore, 1967) but support for this concept came from the rather nebulous observations that fracture of the shell results in heavy contamination of the albumen and a high incidence of rotting (McNally, 1953; Miller and Crawford, 1953; Brown *et al.*, 1966c). Earlier in this discussion it was noted that the shell is perforated with $7–17 \times 10^3$ pores having an average diameter of 9–35 μ, a size that could not be expected to hinder the movement of bacterial cells. Although it has been generally accepted that the cuticle contributes to the shell's resistance to invasion (Haines, 1939; Romanoff and Romanoff, 1949; Brooks and Taylor, 1955), direct evidence has had to await the studies of electron microscopists (Masshoff and Stolpmann, 1961; Simons and Wiertz, 1963, 1965, 1966). Their observations have been summarized in Fig. 2. The cuticle is an organized structure that clothes the surface of the shell. Its surface is irregularly fissured, except when traversing the

TABLE VI

TYPE OF MICROORGANISMS RECOVERED FROM THE SHELL OF THE HEN'S EGG

	Incidence (%) of organisms recovered from the shell of the hen's egg						
		Egg breaking plants[b]			Packing station[c]		
Type of organism	Shops and farms[a]	Clean	Lightly soiled	Heavily soiled	Clean	Lightly soiled	Cracked, etc.
Streptococcus	–	8	5	–	–	–	–
Staphylococcus	5	30	–	–	9	5	11
Micrococcus	18	23	20	–	37	52	42
Sarcina	2	20	–	–	–	–	–
Arthrobacter	–	–	–	–	5	13	10
Bacillus	30	–	13	5	–	2.5	–
Pseudomonas	6	–	–	–	22.5	12.5	24
Achromobacter	19	–	–	–	1.5	2	1
Alcaligenes	–	–	–	–	–	2	–
Flavobacterium	3	–	–	–	–	–	–
Cytophaga	–	–	–	–	–	1	–
Coli-aerogenes	5	19	7	5	10.5	7.5	4
Aeromonas	–	–	–	–	1	–	2
Proteus	1	–	20	20	–	–	–
Serratia	–	–	20	50	–	–	–
Molds	7	–	10	20	–	–	–
Unclassified	–	–	–	–	12[d]	5[d]	6[d]
No. organisms studied	100	NR[e]	NR[e]	NR[e]	130	164	126

[a] Haines (1938).
[b] Zagaevsky and Lutikova (1944).
[c] Board *et al.* (1964).
[d] Aerobic gram-negative bacteria.
[e] Numbers not given in report.

TABLE VII

TYPES OF ORGANISMS RECOVERED FROM CONTENTS OF EGGS

Type of egg	Organisms[a]						
	Coli-aerogenes	*Proteus*	*Aeromonas*	*Pseudomonas*	*Alcaligenes*	*Achromobacter*	Gram+ve bacteria
Rotten eggs							
Haines (1938)	+	+	−	+	+	+	−
Alford *et al.* (1950)	+	+	−	+	+	+	−
Florian and Trussell (1957)	+	+	+	+	+	+	±
Board (1965b)	+	+	+	+	+	+	±
Board and Board (1968)	+	+	+	+	+	+	±
Tainted eggs							
Richard and Mohler (1950)	+	−	−	+	+	+	±
Incubator rejects							
Board and Board (unpublished observations)[b]	±	−	−	−	−	−	Micrococci and staphylococci
Harry (1957)[c]	+	+	−	−	−	−	*Bacillus, micrococcus, streptococcus*

[a] +, Isolated on many occasions; ±, isolated occasionally; −, not isolated.

[b] Results obtained from an investigation of >1000 eggs in which only limited embryological development had occurred; rotten eggs or "dead-in-shells" not included.

[c] Organisms derived from the yolk of "dead-in-shells" or chicks which died shortly after hatching.

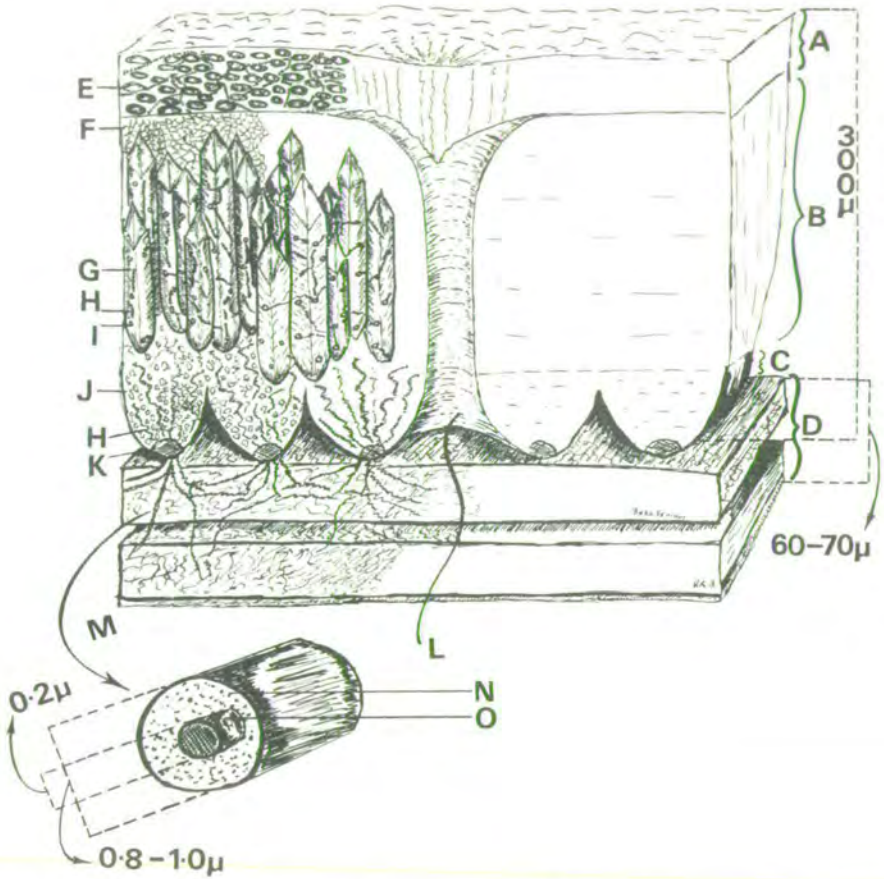

FIG. 2. An artist's impression of the organization of the egg shell as shown by a radial section through a pore. A, Cuticle; B, spongy layer; C, mammillary layer; D, shell membranes; E, vesicles and fibers; F, amorphous crystalline materials; G, columns of calcite crystal; H, fibers; I, vesicles; J, amorphous crystalline material; K, protein plug; L, pore canal; M, large fibers; N, mantle (composition uncertain); O, keratin core.

campanulate orifice of the pores where, presumably due to the stresses arising from drying, the fissures have a radial arrangement. The cuticle is fairly resistant to water or detergents and to gentle rubbing with a cloth (Simons and Wiertz, 1966). It is less resistant to abrasives, and wiping eggs with sandpaper or wire wool causes an increase in (1) the rate of evaporation (Marshall and Cruickshank, 1938; Tyler, 1945), (2) the shell's permeability to dyes (Fromm and Munroe, 1960), and (3) the incidence of rotting (Haines, 1938; Haines and Moran,

1940; Brown *et al.*, 1966a). When egg shells are breached under farm conditions, it is suspected that only ten or so pores provide portals for the entry of microorganisms (Bryant and Sharp, 1934; Orel, 1959). Likewise only a few pores are invaded when eggs are challenged by large numbers of organisms under laboratory conditions (Paton and Ayres, 1964; Board and Board, 1967). How these pores differ from the norm is not known, but the author suspects that they may be exceptionally wide and not capped with cuticle.

Water appears to be an essential agent for microbial invasion of the shell. Thus humid conditions promote the growth of molds at the surface of the shell (Sharp and Stewart, 1936); hyphae penetrate the pores (Weston and Halnan, 1927), and bacteria can be lodged in the shell membranes (Zagaevsky and Lutikova, 1944). When eggs are submerged in water of the same temperature, capillarity causes a flooding of the pore canals (Haines and Moran, 1940). These workers deduced also that water is sucked into the pores when a warm egg contracts in cold water. In view of the observation that an egg, from which a small piece of shell has been removed, is ruptured when placed in water (Lifshitz *et al.*, 1964) it is conceivable that movement of water, and hence microorganisms, through the pores may be accentuated by osmotic forces acting across the semipermeable shell membranes. The mere wiping of a shell with a cloth or brush moistened with a bacterial culture can cause contamination of the egg's contents (Stuart and McNally, 1943; Wilson, 1945; Buxton and Gordon, 1947; Lancaster and Crabb, 1953). It is not clear whether this is due to water being drawn into pores by capillarity or to it being forced (pumped ?) into the orifice of the pores. When any of the above methods have been applied under laboratory or field conditions, it has been found that there is a heavy contamination of the shell membranes and a high incidence of rotting when the eggs are stored (Haines, 1938; Haines and Moran, 1940; Gillespie *et al.*, 1950a). Because the incidence of rotting is highest when eggs are caused to contract in water, this phenomenon has been the subject of many investigations. It has been established that the following factors influence the extent of microbial penetration of the shell: (1) the temperature differential between the egg and the bacterial suspension, the incidence being directly proportional to the difference in temperature over the range 6°–21°C. (Lorenz *et al.*, 1952; Brant and Starr, 1962), (2) the number of organisms in the suspension (Stokes *et al.*, 1956; Hartung and Stadelman, 1963), (3) the period of immersion—an osmotic effect ?—(Hartung and Stadelman, 1962), and (4) the thick-

ness of the shell, thin shells offering less resistance than thick ones (Orel, 1959). From this evidence, it can be inferred that the shell is poorly adapted to function under wet conditions. This is unfortunate since washing is the most convenient method of cleaning eggs for market. Conflicting results came from early studies concerned with the storage behavior of washed eggs, but they did establish that certain designs of egg washing machines tended to increase the incidence of rotting (Gillespie *et al.*, 1950b,c; Trussell *et al.*, 1955a,b; Knowles, 1957a,b). Moreover the use of a disinfectant, although it reduces the level of contamination of the shell (Winter *et al.*, 1952, 1955), does not guarantee freedom from spoilage during the storage of treated eggs (Gillespie *et al.*, 1950c; Sauter *et al.*, 1962; Sauter, 1966). In certain instances the use of a selective disinfectant can cause a relative increase in the number of gram-negative bacteria (Gillespie *et al.*, 1950c), whereas, in others, the organisms penetrate the pores and are protected against inimical agents by the shell membranes (Cotterill and Hartman, 1956; Elliott and Romoser, 1957; Schmidt and Stadelman, 1957; Bean and McLaury, 1959; Rizk *et al.*, 1966a). To overcome these difficulties, many investigations have been concerned with pasteurization of shell eggs; it has been established that deterioration due to microbial action is of rare occurrence in eggs which have been held briefly at temperatures of 135.5°–158°F. (Goresline *et al.*, 1950; Funk, 1943, 1948; Romanoff and Romanoff, 1944; Murphy and Sutton, 1947; Salton *et al.*, 1951; Funk *et al.*, 1954; Scott and Vickery, 1954; Knowles, 1956). In recent years, experience gained through study of microbial invasion of the shell together with that reviewed immediately above has lead to marked improvements in the design and operation of egg washing machines. Of particular importance is the requirement for the wash water to be maintained at a temperature higher than that of the egg. A differential of 20°F. has been recommended by Brant *et al.* (1966) and 20°C. by Büchli (1967). With the latter, the water used for washing and *rinsing* the eggs is held at 40°C., a temperature which may well have a pasteurizing effect. Moreover when alkaline detergents (pH 10) are used at this temperature, the wash water and the machine are kept relatively free of microorganisms (Büchli, 1967) thus permitting the recirculation of water with obvious savings in operating costs.

C. Colonization

It can be assumed that any agent that causes microbial penetration of the shell will result in a *heterogeneous* population being intro-

duced on or near to the shell membranes; organisms have to be lodged within 30–35 μ of the membranes before they meet water activity (aw) (0.98) suitable for growth (Gillespie and Scott, 1950). Lysozyme-sensitive organisms will be destroyed by the enzyme present in the shell membranes (Korotkova, 1957). As such organisms make only a slight contribution to the microflora of the shell (Board, 1968), this component of the egg's defense would appear to be of relatively little significance at this stage in the infection process. When the shell membranes are suspended in a solution of mineral salts, the level of available nutrients supports appreciable growth of the common rot-producing bacteria (Elliott and Brant, 1957; Board, 1965a; Stokes and Osborne, 1956; Garabaldi and Stokes, 1958). Likewise there is significant growth when organisms are placed on the surface of the shell membranes *in ovo* (Board, 1968). When, however, they become enmeshed in the membranes the amount of growth is slight (Brooks, 1960b; Board, 1964). This has been attributed (Board, 1968) to the albumen influencing the environment within the structures. The response of organisms to these conditions has been the subject of limited study only (Board, 1968; J. Roger Saxon and R. G. Board, unpublished observations). These have indicated that the early phase of infection of the membranes is characterized by a selection of organisms. In practice this means that the gram-negative fraction of the flora increases at the expense of the gram-positive one. The actual rate of change in these fractions is determined by temperature and this influences also the composition of the gram-negative fraction. For example, there is evidence that coliforms do not develop in eggs held at room temperature, but do so with storage at 37°C. These initial observations tend toward explaining the previously noted change from a predominantly gram-positive flora of the shell to a gram-negative one in the rotten eggs.

In eggs contaminated under farm or laboratory conditions and held at ambient temperatures, it is notable that, following invasion of the shell, the infection remains confined to the shell membranes for upward of 15–20 days (Zagaevsky and Lutikova, 1944; Gillespie and Scott, 1950; Bigland and Papas, 1953; Miller and Crawford, 1953; Elliott, 1954; Stokes *et al.*, 1956; Orel, 1959; Fromm and Munroe, 1960; Garibaldi and Bayne, 1960; Rizk *et al.*, 1966b; Büchli, 1967). Is it merely a coincidence that this period is slightly longer than that needed by the wild hen to lay a "clutch" of 12 eggs (Harland, 1927)? There has been a tendency to ascribe (Kraft *et al.*, 1958) this lag in the infection process to the membranes imposing a barrier to microbial

movement. Such an interpretation has been prompted, no doubt, by the repeated observation that the shell membranes can be made to act as bacterial filters (Walden *et al.*, 1956; Garibaldi and Stokes, 1958). Under test conditions, however, the membranes are subjected to challenges different from those occurring under practical ones. For example, Haines and Moran (1940) and Garibaldi and Stokes (1958) applied pressure to the outer surface of eggs, the contents of which had been replaced with suspensions of bacteria. It is probable that under these conditions the shell membranes were compressed and forced up against the internal orifice of the pores, thereby increasing resistance to bacterial movement. The results of such studies also show poor agreement with the observations of persons who have used whole eggs. Thus, Bean and McLaury (1959) recovered organisms from the inner surface of the shell membranes immediately following the contraction of a warm egg in a cold suspension of bacteria; Board and Ayres (1965) noted the rapid penetration of the inner shell membrane *in ovo*. This membrane is considered to offer the greatest resistance to bacterial invasion (Lifshitz *et al.*, 1964). Likewise the observations that the mantle surrounding the keratin fibers (Fig. 2) can be removed by bacteria (Brown *et al.*, 1965) does not necessarily imply that they "digest" their way through the membranes. The latter had been in contact with bacteria for 15 days and it is probable that the modification of the mantles occurred when bacteria began to grow in the albumen—a period during which the physiological activity of the organisms is likely to be at a maximum. Moreover, bacteria that release nitrogenous substances from shell membranes *in vitro* do not penetrate the membranes at a rate faster than that of those which have no demonstrable action on these structures (Garibaldi and Stokes, 1958; Board, 1965a). Many factors appear to influence the rate and extent of microbial growth in the shell membranes. With *Serratia marcescens*, for example, no growth occurred when infected eggs were held at 10°C., and it was quickly killed when eggs were stored at 37°C. (Board and Ayres, 1965). At this temperature, coliforms do not die (Ellenor G. H. Wilson and R. G. Board, unpublished observations). When eggs contaminated with serratiae were held at room temperature, slight growth took place in the 2–4 days following inoculation, a response that is typical of the majority of rot-producing bacteria (Board, 1964). With storage at 0°–10°C., psychrotrophic bacteria such as *Pseudomonas fluorescens* grow in the membrane without obvious hindrance (Board and Ayres, 1965). Their growth can be prevented by the storage of infected eggs

in high concentrations of CO_2 (Sally Beastall and R. G. Board, unpublished observations). In contrast, traces of iron at the site of infection of the shell membranes promote extensive microbial growth (Board *et al.*, 1968). The same response is observed when the chelating potential of the conalbumin of the albumen is satisfied (Board, 1964). Under commercial conditions it has been demonstrated that contamination, either deliberate or natural, of wash water with iron can result in an increase in the rate and extent of rotting of stored eggs (Garibaldi and Bayne, 1960, 1962a,b).

This evidence indicates that the fate of contaminants of the shell membranes is determined by an interplay of factors intrinsic (lysozyme and conalbumin) and extrinsic (temperature, gaseous environment, and presence or absence of additional iron) to the egg with those implicit to the organisms themselves. It would appear that this phase of the infection process should be studied in greater detail because a better understanding of their interplay might suggest methods that would ensure the safety not only of eggs intended for human consumption but also those used by the hatching industry.

The growth lag noted previously is apparent when relatively large numbers of organisms are placed directly in the albumen (Brown *et al.*, 1966b). Moreover, this lag is not appreciably influenced even when large numbers of organisms are placed on the shell membranes. Thus both Brooks (1960b) and Board (1964) noted a lag of ca. 13 days, although the former used an inoculum 100–1000 times greater than the latter. Moreover, Brooks noted that the albumen was contaminated shortly after the seeding of the shell membranes. There is much indirect evidence that the early contaminants of the albumen fail to multiply and that they may even be killed. This feature has not been studied in detail but the available evidence suggests that the fate of an organism is determined by the temperature of incubation. Thus, it is noteworthy that Sharp and Whitaker (1927) achieved rapid killing by placing young cells in albumen (*in vitro*) at 37°C., whereas Garibaldi (1960), who used essentially the same range of organisms, observed only a slight decline in the numbers of viable organisms when 18-hour cultures were used to seed albumen. With infected, whole eggs held at 10°C., Board and Ayres (1965) noted a progressive build-up in the contaminants of the albumen. Although the evidence is fragmentary, it is suspected that the antimicrobial defense of the albumen decreases in efficiency as the temperature moves away from that of the hen. This feature is worthy of further exploration since the results might indicate means whereby the albumen of commerce

could be freed of microorganisms without impairing its functional properties.

The cardinal importance of the albumen in the egg's defense was recognized by Sharp and Whitaker (1927), and they concluded that actual rotting did not occur until organisms made contact with the yolk. This mode of induction was suggested also by Board and Ayres (1965) to account for sudden macroscopic changes in eggs the albumen of which became progressively contaminated during storage at 10°C. These observations suggest that the viscosity of the albumen and the gelatinous nature of the albuminous sac are important components of the egg's defense since they impede microbial movement. This accounts, no doubt, for the observations (Gillespie and Scott, 1950) that the initial contaminants of the albumen occur in clumps, a phenomenon that is particularly notable early in the infection of eggs with fluorescent pseudomonads. The observations of Brooks (1960b), Board (1964), and Board and Ayres (1965) suggest that when eggs are held at room temperature or above, addling begins with the growth of organisms that have reached the junction of the yolk and shell membranes. In other words, the antimicrobial defense system of the albumen is short-circuited by the yolk touching the membranes. Is it for this reason that selection favored hens that turned their eggs during incubation? Regular turning maintains tension in the chalazae, thereby ensuring that the yolk is retained in a central position. Little is known concerning the fate of the initial contaminants of the albumen. Earlier in the discussion it was inferred that they remain quiescent. Such a view receives support from the observations that there are no demonstrable changes in the hydrogen ion, glucose (Board, 1964; Board *et al.*, 1968), or amino acid (Dr. D. J. Stewart, personal communication) content of the albumen until there is visible evidence of infection. At this time there is active multiplication of organisms in the albumen and population of 10^9 organisms/ml. albumen are achieved.

With eggs contaminated under commercial conditions, it is usual for a mixed population of gram-negative organisms to be present in the contents when rotting occurs (Board, 1965b; Board and Board, 1968). This suggests that the selective influences present in the shell membranes occur in an accentuated form in the albumen also. Following the investigations of Miles and Halnan (1937) it became customary to seed fresh eggs with cultures obtained from rots. The organism that produced changes similar to those observed in the original rot was referred to as the rot-producer, whereas, the others were considered to

be adventitious contaminants (Haines, 1938) or secondary invaders (Florian and Trussell, 1957). It is noteworthy that the course of infection in eggs inoculated with the former is essentially the same as that of the latter (Board, 1964). The main difference between the two groups resides in the metabolic attributes of the rot-producing organisms. They possess one or more of the following properties (Table VIII): (1) production of a pigment, (2) digestion of protein with or without H_2S production, and (3) an ability to attack lecithin. On occasion an egg can be infected with organisms which do not have these attributes, and such an egg appears normal in a cursory examination but it can be tainted (Richard and Mohler, 1950). This indicates that the terms "adventitious contaminant" or "secondary invaders" are of

TABLE VIII

METABOLIC ATTRIBUTES OF COMMON CONTAMINANTS OF ADDLED EGGS AND THEIR ROLE IN THE ROTTING PROCESS[a]

Attribute	Action	Organisms[b]
Nonwater soluble pigment	Discoloration of shell membrane at site of infection, occasionally in the white and on the surface of the yolk	*Cytophaga, Flavobacterium, Serratia*
Water-soluble pigment	Discoloration of white	*Pseudomonas aeruginosa, P. putida,* and *P. fluorescens*
Proteolysis	Digestion of white and yolk	*Proteus, Aeromonas*
Production of H_2S	Blackening of the yolk	*Proteus, Aeromonas*
Lecithinase	Breakdown of yolk emulsion	*cloacae*
Slime production	Increase in viscosity of the albumen	*cloacae* (some strains only)
Odor production	Characteristic odor emitted by infected eggs	*Pseudomonas maltophilia*
None of the above	No macroscopic changes even when eggs harbor 10^8 organisms/ml. albumen	*Alcaligenes, Salmonella, Citrobacter*

[a] Table compiled from data given by Haines (1939), Platt and Anderson (1939), Florian and Trussell (1957), and Board and Board (1968).

[b] Some of the organisms have several of the metabolic attributes but it is usual for the changes in an egg to be dominated by the activity of one attribute only.

little relevence and it would appear they could be replaced by the terms used in ecology. Thus, the populations of rotten eggs can be considered to consist of three fractions: (1) the dominant (rot- or taint-producing) organisms, (2) the associates (those organisms which fail to produce significant changes in the egg), and (3) the incidentals (i.e., gram-positive bacteria such as streptococci and micrococci which are occasionally present in low numbers only).

In this discussion of the course of the infection process, emphasis has been given to the integrated workings of the shell, the shell membranes, and the albumen in the egg's defense. When the egg is considered as an ecosystem it can be argued that the albumen plays the principal role in isolating the blastoderm and its food supply from the environment of the hen. In other words, it provides an aqueous phase in which embryo development can occur without direct competition from other organisms. As yet we have only an incomplete picture of the role of the majority of components of the albumen. This would appear to be a subject worthy of further investigation especially since the "loss" due to low hatchability costs the British poultry industry more than £1 million per annum (Mr. A. E. Beer, personal communication).

Addendum

Can we now, with the cogency borne of hindsight, offer an explanation of the real basis of the argument twixt Donné and Pasteur? The author suggests that the passion of dogma may have caused Donné to shake violently his eggs thereby rupturing the yolk membranes and negating the antimicrobial defense whereas Pasteur, with sagacious gentleness, maintained the integrity of the egg.

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References

Abels, J. C. (1936). *J. Am. Chem. Soc.* **58**, 2609–2610.
Aisen, P., Leibman, A., and Reich, H. A. (1966). *J. Biol. Chem.* **241**, 1666–1671.
Alderton, G., Ward, W. H., and Fevold, H. L. (1946). *Arch. Biochem.* **11**, 9–13.
Alford, L. R., Holmes, N. E., Scott, W. J., and Vickery, J. R. (1950). *Australian J. Appl. Sci.* **I**, 208–214.
Asbell, M. A., and Eagon, R. G. (1966). *Biochem. Biophys. Res. Commun.* **22**, 664–671.
Ayres, J. C., and Taylor, B. (1956). *Appl. Microbiol.* **4**, 355–359.
Azari, P. R., and Baugh, R. F. (1967). *Arch. Biochem. Biophys.* **118**, 138–144.

Azari, P. R., and Feeney, R. E. (1958). *J. Biol. Chem.* **232,** 293–302.
Azari, P. R., and Feeney, R. E. (1961). *Arch. Biochem. Biophys.* **92,** 44–52.
Bain, J. A., and Deutsch, H. F. (1948). *J. Biol. Chem.* **172,** 547–555.
Baker, C. M. A. (1960). *Brit. Poultry Sci.* **1,** 3–16.
Baker, C. M. A. (1968). *In* "Egg Quantity: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 67. Oliver & Boyd, Edinburgh.
Baker, C. M. A., and Manwell, C. (1962). *Brit. Poultry Sci.* **3,** 161–174.
Baker, R. C., and Stadelman, W. J. (1958). *Poultry Sci.* **37,** 558–564.
Baskett, R. G., Dryden, W. H., and Hale, R. W. (1937). *J. Min. Agr. Northern Ireland* **5,** 132–142.
Bauchop, T., and Elsden, S. R. (1960). *J. Gen. Microbiol.* **23,** 457–469.
Bean, K. C., and McLaury, D. W. (1959). *Poultry Sci.* **38,** 693–698.
Beer, A. E. (1967). *Poultry Rev.* **6,** 70–75.
Berger, L. P., and Weiser, R. S. (1957). *Biochim. Biophys. Acta* **26,** 517–521.
Bigland, C. H., and Papas, G. (1953). *J. Comp. Med.* **17,** 105–109.
Birdsell, D. C., and Cota-Robles, E. H. (1967). *J. Bacteriol.* **93,** 427–437.
Board, P. A., and Board, R. G. (1967). *Lab. Practice* **16,** 471–482.
Board, P. A., and Board, R. G. (1968). *Brit. Poultry Sci.* **9,** 111–120.
Board, P. A., Hendon, L. P., and Board, R. G. (1968). *Brit. Poultry Sci.* **9,** 211–215.
Board, R. G. (1964). *J. Appl. Bacteriol.* **27,** 350–364.
Board, R. G. (1965a). *J. Appl. Bacteriol.* **28,** 197–205.
Board, R. G. (1965b). *J. Appl. Bacteriol.* **28,** 437–453.
Board, R. G. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 133. Oliver & Boyd, Edinburgh.
Board, R. G., and Ayres, J. C. (1965). *Appl. Microbiol.* **13,** 358–364.
Board, R. G., and Wilson, E. G. H. (1965). *Edinburgh School Agr. Exptl. Work,* 89–90.
Board, R. G., Ayres, J. C., Kraft, A. A., and Forsythe, R. H. (1964). *Poultry Sci.* **43,** 584–594.
Brambell, F. W. R. (1958). *Biol. Rev.* **33,** 488–531.
Brandley, C. A., Moser, H. E., and Jungherr, E. L. (1946). *Poultry Sci.* **25,** 397–398.
Brant, A. W., and Starr, P. B. (1962). *Poultry Sci.* **41,** 1468–1473.
Brant, A. W., Starr, P. B., and Hamaan, J. A. (1966). *Poultry Tribune,* p. 65.
Brooks, J. (1960a). *In* "Texture in Foods." Soc. Chem. Inc., London.
Brooks, J. (1960b). *J. Appl. Bacteriol.* **23,** 499–509.
Brooks, J., and Hale, H. P. (1959). *Biochim. Biophys. Acta* **32,** 237–250.
Brooks, J., and Taylor, D. J. (1955). Eggs and Egg Products. *Rept. Food Invest. Bd. No.* **60.** HMSO, London.
Brown, W. E., Baker, R. C., and Naylor, H. B. (1965). *Poultry Sci.* **44,** 1323–1327.
Brown, W. E., Baker, R. C., and Naylor, H. B. (1966a). *Poultry Sci.* **45,** 276–279.
Brown, W. E., Baker, R. C., and Naylor, H. B. (1966b). *Poultry Sci.* **45,** 279–283.
Brown, W. E., Baker, R. C., and Naylor, H. B. (1966c). *Poultry Sci.* **45,** 284–287.
Brumfitt, W. (1959). *Brit. J. Exptl. Pathol.* **40,** 441.
Bryant, R. L., and Sharp, P. L. (1934). *J. Agr. Res.* **48,** 67–89.
Büchli, K. (1967). *Inst. Pluimveeteelt 'Het Spelderholt'—Beekbergen Mededel. No.* **145.**
Burge, R. E., and Draper, J. C. (1967a). *J. Mol. Biol.* **28,** 173–187.
Burge, R. E., and Draper, J. C. (1967b). *J. Mol. Biol.* **28,** 189–204.
Burge, R. E., and Draper, J. C. (1967c). *J. Mol. Biol.* **28,** 205–210.
Buxton, A., and Gordon, R. F. (1947). *J. Hyg.* **45,** 265–281.

Carey, N. H. (1966). *In* "Physiology of the Domestic Fowl" (C. Horton-Smith and E. C. Amoroso, eds.), p. 125–132. Oliver and Boyd, Edinburgh.
Caroline, L., Taschdjian, C. L., Kozinn, P. J., and Schade, A. L. (1964). *J. Invest. Dermatol.* **42,** 415–419.
Carson, K. J., and Eagon, R. G. (1966). *Can. J. Microbiol.* **12,** 105–108.
Clark, J. R., Osuga, D. T., and Feeney, R. E. (1963). *J. Biol. Chem.* **238,** 3621–3631.
Collins, F. M., and Lascelles, J. (1962). *J. Gen. Microbiol.* **29,** 531–535.
Conti, S. F., Jacobs, N. J., and Gray, C. T. (1968). *J. Bacteriol.* **96,** 554–556.
Cotterill, O. J., and Gardner, F. (1957). *Poultry Sci.* **36,** 196–206.
Cotterill, O. J., and Hartman, P. (1956). *Poultry Sci.* **35,** 733–735.
Cotterill, O. J., and Winter, A. R. (1954a). *Poultry Sci.* **33,** 607–611.
Cotterill, O. J., and Winter, A. R. (1954b). *Poultry Sci.* **33,** 679–686.
Cotterill, O. J., and Winter, A. R. (1955). *Poultry Sci.* **34,** 679–686.
Cotterill, O. J., Gardner, F. A., Funk, E. M., and Cunningham, F. E. (1958). *Poultry Sci.* **37,** 479–483.
Cox, S. T., Jr., and Asbell, R. G. (1968). *Can. J. Microbiol.* **14,** 913–922.
Cunningham, F. E., and Lineweaver, H. (1965). *Food Technol.* **19,** 136–141.
Davis, B. D. (1961). *Cold Spring Harbor Symp. Quant. Biol.* **26,** 1–10.
Davis, B., Saltman, P., and Benson, S. (1962). *Biochem. Biophys. Res. Commun.* **8,** 56.
Dean, A. C. R., and Hinshelwood, C. (1966). "Growth Function and Regulation in Bacterial Cells." Clarendon Press, Oxford.
Delezenne, C., and Pozerski, E. (1903). *Compt. Rend. Soc. Biol.* **55,** 935.
Ducay, E. D., Kline, L., and Mandeles, S. (1960). *Poultry Sci.* **39,** 831–835.
Eakin, R. E., Snell, E. E., and Williams, R. J. (1940). *J. Biol. Chem.* **136,** 801–802.
Ehrenberg, A., and Laurell, C. B. (1955). *Acta Chem. Scand.* **9,** 68–72.
Elliott, R. P. (1954). *Appl. Microbiol.* **2,** 158–163.
Elliott, R. P., and Brant, A. W. (1957). *Food Res.* **22,** 241–250.
Elliott, R. P., and Romoser, G. L. (1957). *Poultry Sci.* **36,** 365–375.
Evans, R. J., Davidson, J. A., and Butts, H. A. (1950). *Poultry Sci.* **29,** 104–108.
Evans, R. J., Butts, H. A., and Davidson, J. A. (1951). *Poultry Sci.* **30,** 515–519.
Feeney, R. E. (1951). *Arch. Biochem. Biophys.* **34,** 196–208.
Feeney, R. E., and Nagy, D. A. (1952). *J. Bacteriol.* **64,** 629–643.
Feeney, R. E., Ducay, E. D., Silva, R. S., and MacDonnell, C. R. (1952). *Poultry Sci.* **31,** 639–647.
Feeney, R. E., Weaver, J. M., Jones, J. R., and Rhodes, M. B. (1956). *Poultry Sci.* **35,** 1061–1066.
Feeney, R. E., Stevens, F. C., and Osuga, D. T. (1963). *J. Biol. Chem.* **238,** 1415–1418.
Fleming, A. (1922). *Proc. Roy. Soc.* **B93,** 306–317.
Florian, M. L. E., and Trussell, P. C. (1957). *Food Technol.* **11,** 56–60.
Forsythe, R. H., Ayres, J. C., and Radlo, J. L. (1953). *Food. Technol.* **7,** 49–56.
Fossum, K., and Whitaker, J. R. (1968). *Arch. Biochem. Biophys.* **125,** 367–375.
Fraenkel-Conrat, H. L., and Feeney, R. E. (1950). *Arch. Biochem.* **29,** 101–113.
Frazer, D. T., Jukes, T. H., Branion, H. D., and Halpen, K. C. (1934). *J. Immunol.* **26,** 437–446.
Friedberger, E., and Hoder, F. (1932). *Z. Immunitaetforsch.* **74,** 429–447.
Fromm, D. (1967). *J. Food Sci.* **32,** 1–5.
Fromm, D., and Monroe, R. J. (1960). *Food Technol.* **14,** 401–403.
Fuller, R. A., and Briggs, D. R. (1956). *J. Am. Chem. Soc.* **78,** 5253–5257.
Fuller, R., and Jayne Williams, D. J. (1968). *Brit. Poultry Sci.* **9,** 159–163.

Funk, E. M. (1943). *Res. Bull. Missouri Agr. Exptl. Stat. No.* **364.**
Funk, E. M. (1948). *Res. Bull. Missouri Agr. Exptl. Stat. No.* **426.**
Funk, E. M., Forward, J., and Lorah, M. (1954). *Res. Bull. Missouri Agr. Exptl. Stat. No.* **550.**
Gardner, J. F., and Lascelles, J. (1962). *J. Gen. Microbiol.* **29,** 157–164.
Garibaldi, J. A. (1960). *Food Res.* **25,** 337–344.
Garibaldi, J. A. (1967). *J. Bacteriol.* **94,** 1296–1299.
Garibaldi, J. A., and Bayne, H. G. (1960). *Poultry Sci.* **39,** 1517–1520.
Garibaldi, J. A., and Bayne, H. G. (1962a). *J. Food Sci.* **27,** 57–59.
Garibaldi, J. A., and Bayne, H. G. (1962b). *Poultry Sci.* **41,** 850–853.
Garibaldi, J. A., and Neilands, J. B. (1956). *Nature* **177,** 526–527.
Garibaldi, J. A., and Stokes, J. L. (1958). *Food Res.* **23,** 283–290.
Garrard, W., and Lascelles, J. (1968). *J. Bacteriol.* **95,** 152–156.
Gayon, M. V. (1873). *Compt. Rend.* **76,** 232–233.
Ghosh, B. K., and Murray, R. G. E. (1967). *J. Bacteriol.* **93,** 411–426.
Gillespie, J. H., and Scott, W. J. (1950). *Australian J. Appl. Sci.* **1,** 514–530.
Gillespie, J. H., Scott, W. J., and Vickery, J. R. (1950a). *Australian J. Appl. Sci.* **1,** 215–223.
Gillespie, J. H., Scott, W. J., and Vickery, J. R. (1950b). *Australian J. Appl. Sci.* **1,** 313–329.
Gillespie, J. H., Salton, M. R. J., and Scott, W. J. (1950c). *Australian J. Appl. Sci.* **1,** 531–538.
Good, R. A., Finstad, J., Gewurz, H., Cooper, M. D., and Pollara, B. (1967). *Am. J. Diseases Children* **114,** 477–497.
Gordon, R. F., and Tucker, J. F. (1954). *Contrib. 109 10th World's Poult. Congr.* Edinburgh, Department of Agriculture, Scotland.
Goresline, H. E., Moser, R. E., and Hayes, K. M. (1950). *Food Technol.* **4,** 426–430.
Gray, C. T., Wimpenney, J. W. T., Hughes, D. E., and Mossman, M. R. (1966a). *Biochim. Biophys. Acta* **117,** 22–32.
Gray, C. T., Wimpenney, J. W. T., and Mossman, M. R. (1966b). *Biochim. Biophys. Acta* **117,** 33–41.
Gray, G. W., and Wilkinson, S. G. (1965a). *J. Appl. Bacteriol.* **28,** 153–164.
Gray, G. W., and Wilkinson, S. G. (1965b). *J. Gen. Microbiol.* **39,** 385–389.
Haines, R. B. (1938). *J. Hyg.* **38,** 338–355.
Haines, R. B. (1939). *Rept. Food Invest. Bd. No.* **47.** HMSO, London.
Haines, R. B., and Moran, T. (1940). *J. Hyg.* **40,** 453–461.
Hale, H. P. (1950). *J. Sci. Food Agr.* **1,** 46–48.
Halkett, J. A. E., Peters, T., and Ross, J. F. (1958). *J. Biol. Chem.* **231,** 187–199.
Hara, S., and Matsushima, Y. (1967). *J. Biochem.* **62,** 118–125.
Harland, H. C. (1927). *J. Genet.* **18,** 55–62.
Harry, E. G. (1957). *Vet. Record* **69,** 1433–1439.
Harry, E. G. (1963a). *Brit. Poultry Sci.* **4,** 63–70.
Harry, E. G. (1963b). *Brit. Poultry Sci.* **4,** 91–100.
Hartung, T. E., and Stadelman, W. J. (1962). *Poultry Sci.* **41,** 1590–1591.
Hartung, T. E., and Stadelman, W. J. (1963). *Poultry Sci.* **42,** 147–150.
Hirsch, J. G. (1965). *Ann. Rev. Microbiol.* **19,** 339–350.
Jacobs, N. J., and Conti, S. F. (1965). *J. Bacteriol.* **89,** 675–679.
Karminiski, M., and Durieux, J. (1956). *Exptl. Cell Res.* **10,** 590–618.
Kellenberger, E., and Ryter, A. (1958). *J. Biophys. Biochem. Cytol.* **4,** 323–326.

Keller, W., and Pennell, R. B. (1958). *J. Lab. Clin. Med.* **53,** 638–645.
King, J. V., Campbell, J. J. R., and Eagles, B. A. (1948). *Can. J. Res.* **26,** 514–519.
Knowles, N. R. (1956). *J. Appl. Bacteriol.* **19,** 293–300.
Knowles, N. R. (1957a). *Res. Exptl. Record Min. Agr. Northern Ireland* **6,** 100–107.
Knowles, N. R. (1957b). *Res. Exptl. Record Min. Agr. Northern Ireland* **6,** 108–117.
Kohn, A. (1960). *J. Bacteriol.* **79,** 697–706.
Kraft, A. A., Elliott, L. E., and Brant, A. W. (1958). *Poultry Sci.* **37,** 238–240.
Korotkova, G. P. (1957). *Zh. Obshch. Biol.* **18,** 275–287.
Lack, D. (1968). "Ecological Adaptions for Breeding in Birds." Methuen, London.
Lancaster, J. E., and Crabb, W. E. (1953). *Brit. Vet. J.* **109,** 139–148.
Laurell, C. B., and Ingleman, B. (1947). *Acta Chem. Scand.* **1,** 770.
Laschtschenko, P. (1909). *Z. Hyg. Infektionskrankh.* **64,** 419–427.
Lifshitz, A., Baker, R. C., and Naylor, H. B. (1964). *J. Food Sci.* **29,** 94–99.
Longsworth, L. G., Cannan, R. K., and MacInnes, D. A. (1940). *J. Am. Chem. Soc.* **62,** 2580–2590.
Lorenz, F. W., Starr, P. B., Starr, M. P., and Ogasawara, F. X. (1952). *Food Res.* **17,** 351–360.
Lush, I. E. (1961). *Nature* **189,** 981–984.
McNally, E. H. (1953). *Poultry Sci.* **32,** 915.
Magwood, S. E. (1964a). *Poultry Sci.* **43,** 441–449.
Magwood, S. E. (1964b). *Poultry Sci.* **43,** 1567–1572.
Magwood, S. E., and Marr, H. (1964). *Poultry Sci.* **43,** 1558–1566.
Mandeles, S., and Ducay, E. D. (1961). *J. Biol. Chem.* **237,** 3196–3199.
Mandelstam, J., and Rogers, H. J. (1959). *Biochem. J.* **72,** 654.
Manwell, C. (1963). *In* "The Biology of Myxine," (A. Brodal, and R. Fange, eds.), p. 372–455. Universitetsforloget, Oslo.
Manwell, C. (1967). *Comp. Biochem. Physiol.* **23,** 383–406.
Marshall, M. E., and Deutsch, H. F. (1951). *J. Biol. Chem.* **189,** 1–9.
Marshall, W., and Cruikshank, D. B. (1938). *J. Agr. Sci.* **28,** 24–42.
Martin, H. H. (1963). *J. Theoret. Biol.* **5,** 1–34.
Massoff, W., and Stolphann, H. J. (1961). *Z. Zellforsch.* **55,** 818–832.
Matsushima, K. (1958). *Science* **127,** 1178–1179.
Mellor, D. B., and Banwart, G. J. (1965). *Poultry Sci.* **44,** 1244–1248.
Miles, A. A., and Halnan, E. T. (1937). *J. Hyg.* **37,** 79–97.
Miller, W. A., and Crawford, L. B. (1953). *Poultry Sci.* **32,** 303–309.
Morton, J. R., Gilmour, D. G., McDermid, K. M., and Ogden, A. L. (1965). *Genetics* **51,** 97–107.
Murphy, T. W., and Sutton, W. S. (1947). *Agr. Gaz. N. S. Wales Misc. Publ. No.* **3317.**
Murray, M. W., and Rutherford, P. P. (1963). *Poultry Sci.* **42,** 505–508.
Nermut, M. V., and Murray, R. G. E. (1967). *J. Bacteriol.* **93,** 1949–1965.
Nichols, A. A., Leaver, C. W., and Paynes, J. J. (1967). *Brit. Poultry Sci.* **8,** 297–310.
Ogden, A. L. (1961). *Animal Breeding Abstr.* **29,** 127–138.
Ogden, A. L., Morton, J. R., Gilmour, D. G., and McDermid, E. M. (1962). *Nature* **195,** 1026–1028.
Orel, V. (1959). *Poultry Sci.* **38,** 8–12.
Osborne, T. B., and Cambell, F. (1900). *J. Am. Chem. Soc.* **22,** 422–450.
Park, J. T., and Griffith, M. (1964). *Proc. 3rd Intern. Symp. Fleming's Lysozyme,* Session 1a, p. 23.

Parkinson, T. L. (1966). *J. Sci. Food Agr.* **17,** 101–111.
Paton, A. M. (1959). *Nature* **184,** 1254–1255.
Paton, A. M., and Ayres, J. C. (1964). *Nature* **204,** 803–804.
Patterson, R., Youngner, J. S., Weigle, W. O., and Dixon, F. J. (1962). *J. Immunol.* **89,** 272–278.
Perkins, H. R., and Rogers, H. J. (1959). *Biochem. J.* **72,** 647–654.
Phelps, R. A., Shenstone, F. S., Kemmerer, A. R., and Evans, R. J. (1965). *Poultry Sci.* **44,** 388–394.
Platt, A. E., and Anderson, C. F. (1939). *J. Dept. Agr. S. Australia* **42,** 1040–1041.
Ramsey, H. H., and Wilson, T. E. (1957). *Antonie van Leeuwenhoek J. Microbial. Serol.* **23,** 226.
Repaske, R. (1956). *Biochim. Biophys. Acta* **22,** 189–191.
Repaske, R. (1958). *Biochim. Biophys. Acta* **30,** 225–232.
Rhodes, M. B., Bennett, N., and Feeney, R. E. (1959). *J. Biol. Chem.* **234,** 2054–2060.
Rhodes, M. E., Bennett, N., and Feeney, R. E. (1960). *J. Biol. Chem.* **235,** 1040–1041.
Richard, O., and Mohler, A. (1950). *Mitt. Gebiete Lebensm. Hyg.* **41,** 168–180.
Rizk, S. S., Ayres, J. C., and Kraft, A. A. (1966a). *Poultry Sci.* **45,** 764–769.
Rizk, S. S., Ayres, J. C., and Kraft, A. A. (1966b). *Poultry Sci.* **45,** 825–829.
Rogers, H. J. (1965). *In* "Function and Structure in Micro-organisms" (M. R. Pollock and M. H. Richmond, eds.), Symp. Soc. Gen. Microbiol., No. 15, pp. 186–219. Cambridge Univ. Press, Cambridge.
Rogers, H. J., and Perkins, H. R. (1968). "Cell Wall Membranes." Spon, London.
Romanoff, A. L. (1967). "Biochemistry of the Avian Embryo." Wiley (Interscience), New York.
Romanoff, A. L., and Romanoff, A. J. (1944). *Food Res.* **9,** 358–366.
Romanoff, A. L., and Romanoff, A. J. (1949). "The Avian Egg." Wiley, New York.
Rosser, F. T. (1942). *Can. J. Res.* **20D,** 291–296.
Rutherford, P. P., and Murray, M. W. (1963). *Poultry Sci.* **42,** 499–505.
Salton, M. R. J. (1964). "The Bacterial Cell Wall." Elsevier, Amsterdam.
Salton, M. R. J., and Ghuysen, J. M. (1960). *Biochim. Biophys. Acta* **45,** 355–363.
Salton, M. R. J., Scott, W. J., and Vickery, J. R. (1951). *Australian J. Appl. Sci.* **2,** 205–222.
Sauter, E. A. (1966). *Poultry Sci.* **45,** 131–135.
Sauter, E. A., Petersen, C. F., and Lampman, C. E. (1962). *Poultry Sci.* **41,** 468–473.
Schade, A. L. (1958). *Abstr. Proc. 4th Intern. Congr. Biochem. Vienna,* p. 127, 10–42.
Schade, A. L. (1960). *In* "Protides of the Biological Fluids" (H. Peeters, ed, p. 261–263. Elsevier, London.
Schade, A. L. (1963). *Biochem. Z.* **338,** 140–148.
Schade, A. L., and Caroline, L. (1944). *Science* **100,** 14–15.
Schade, A. L., Reinhart, R. W., and Levy, H. (1949). *Arch. Biochem.* **20,** 170–172.
Schade, A. L., Myers, N. H., and Reinhart, R. W. (1968). *J. Gen. Microbiol.* **52,** 253–260.
Schaible, F. J., Bandemer, S. L., and Davidson, J. A. (1946). *Poultry Sci.* **25,** 440–445.
Schmidt, F. J., and Stadelman, W. J. (1957). *Poultry Sci.* **36,** 1023–1026.
Scott, W. J., and Vickery, J. R. (1954). *Australian J. Appl. Sci.* **5,** 89–102.
Sharp, P. F., and Stewart, G. F. (1936). *Mem. Cornell Univ. Agr. Expt. Stat. No.* **91.**
Sharp, P. F., and Whitaker, R. (1927). *J. Bacteriol.* **14,** 17–46.
Shenstone, F. S. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.). Oliver & Boyd, Edinburgh.

Simkiss, K. (1961). *Biol. Rev.* **36,** 321–367.
Simkiss, K. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 3. Oliver & Boyd, Edinburgh.
Simons, P. C. M., and Wiertz, G. (1963). *Z. Zellforsch. Mikroskop. Anat. Histochem.* **59,** 555–567.
Simons, P. C. M., and Wiertz, G. (1965). *Brit. Poultry Sci.* **6,** 283–286.
Simons, P. C. M., and Wiertz, G. (1966). *Poultry Sci.* **45,** 1153–1162.
Simons, P. C. M., Tyler, C., and Thomas, H. P. (1966). *Brit. Poultry Sci.* **7,** 309–314.
Smith, A. J. M. (1934). *J. Exptl. Biol.* **11,** 228–242.
Stokes, J. L., and Osborne, W. W. (1956). *Food Res.* **21,** 264–269.
Stokes, J. L., Osborne, W. W., and Bayne, H. G. (1956). *Food Res.* **21,** 510–518.
Strasters, K. C., and Winkler, K. C. (1963). *J. Gen. Microbiol.* **33,** 213–229.
Strominger, J. L. (1962). *In* "The Bacteria," (I. C. Gunsalus and R. Y. Stanier, eds.), Vol. 3, p. 413. Academic Press, New York.
Stuart, L. S., and McNally, E. H. (1943). *U.S. Egg Poultry Mag.* **49,** 28–31, 45–47.
Summers, D. F., and Hasenclever, H. F. (1964). *J. Bacteriol.* **87,** 1–7.
Sword, C. P. (1966). *J. Bacteriol.* **92,** 536–542.
Symposium, (1967). *Proc. Roy. Soc. (London)* **B167.**
Theodore, T. S., and Schade, A. L. (1965a). *J. Gen. Microbiol.* **39,** 75–83.
Theodore, T. S., and Schade, A. L. (1965b). *J. Gen. Microbiol.* **40,** 385–396.
Tokin, B. P. (1959). *Proc. 15th Intern. Congr. Zool. London.*
Totter, J. R., and Moseley, F. T. (1953). *J. Bacteriol.* **65,** 45–47.
Trussell, P. C., Fulton, C. D., and Cameron, C. I. (1955a). *Food Technol.* **9,** 130–134.
Trussell, P. C., Triggs, R. E., and Green, B. A. (1955b). *Food Technol.* **9,** 134–137.
Tyler, C. (1945). *J. Agr. Sci. Camb.* **36,** 111–116.
Tyler, C. (1953). *J. Sci. Food Agr.* **4,** 266–272.
Tyler, C. (1955). *J. Sci. Food Agr.* **6,** 170–176.
Tyler, C. (1956). *J. Sci. Food Agr.* **7,** 483–493.
Tyler, C. (1968). *Brit. Poultry Sci.* **9,** 143–158.
Vos, J. C. (1964). *J. Gen. Microbiol.* **35,** 313–317.
Vos, J. C. (1967). *J. Gen. Microbiol.* **48,** 391–400.
Walden, C. C., Allen, I. V. F., and Trussell, P. C. (1956). *Poultry Sci.* **35,** 1190–1196.
Warner, R. C., and Weber, I. (1951). *J. Biol. Chem.* **191,** 173–180.
Weibull, C. (1953a). *J. Bacteriol.* **66,** 688–695.
Weibull, C. (1953b). *J. Bacteriol.* **66,** 696–702.
Weidel, W., and Pelzer, H. (1964). *Advan. Enzymol.* **26,** 193–232.
Weinbaum, G., and Markham, R. (1966). *Biochim. Biophys. Acta* **124,** 207–209.
Weibaum, G., Rich, R., and Fischman, D. A. (1967). *J. Bacteriol.* **93,** 1693–1698.
Wells, R. G. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 207. Oliver & Boyd, Edinburgh.
Weston, W. A. R. D., and Halnan, E. T. (1927). *Poultry Sci.* **6,** 251–258.
Williams, J. (1962). *Biochem. J.* **83,** 355–364.
Williams, J. E., and Whittemore, A. D. (1967). *Avian Diseases* **11,** 467–490.
Wilson, J. E. (1945). *Vet. Record* **57,** 411.
Winter, A. R., Burhart, B., and Wettling, C. (1952). *Bull. Ohio Agr. Exp. Stat. No.* **710.**
Winter, A. R., Burhart, B., Clements, P., and MacDonald, L. (1955). *Bull. Ohio Agr. Expt. Stat. No.* **762.**
Wolin, M. J. (1966). *J. Bacteriol.* **91,** 1781–1786.

Wolk, J., McNally, E. H., and Spicknell, N. H. (1950). *Food Technol.* **4,** 316–318.
Woolley, D. W., and Longsworth, L. G. (1942). *J. Biol. Chem.* **142,** 285–290.
Work, E. (1957). *Nature* **179,** 841–847.
Zagaevsky, J. S., and Lutikova, P. O. (1944). *U.S. Egg Poultry Mag.* **50,** 17–20, 43–46, 75–77, 89–90, 121–123.
Zinder, N. D., and Arndt, W. F. (1956). *Proc. Natl. Acad. Sci. U.S.* **42,** 586–590.

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Microbiology of the Egg: A Review

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bution. In addition attention has been given to those unresolved problems that appear to impede further technological innovations in the marketing of shell eggs or handling of egg products.

Contamination of the egg

Before laying. The incidence and level of microbial contamination of the contents of eggs at the time of laying is not known. This is due partly to the technical difficulties associated with sampling the yolk and white under strictly aseptic conditions. Thus no success has attended attempts to find methods that ensure the sterilization of the shell of all eggs (Gillespie & Scott, 1950; Wolk, McNally & Spicknall, 1950; Ayres & Taylor, 1956). Even if this could be achieved, the contents, because of their viscosity, cannot be sampled under conditions which can be considered to exclude possible contamination with airborne organisms. But even if all these problems could be overcome, there would still remain another reason for caution. Many workers have claimed that the shell is easily invaded following laying (Ferdinadov, 1944; Lorenz, Starr, Starr & Ogasawara, 1952; Graves & MacLaury, 1962; Dr J. Patton, personal communication) and that the degree of contamination of the contents of fresh eggs is related directly to the porosity of the shell (Kraft, McNally & Brant, 1958). When reviewing this facet of egg microbiology Brooks & Taylor (1955) were forced to generalize that 'roughly 90% of newly laid eggs are free from microorganisms and the true value may be even higher'.

The commonest contaminants of the contents of fresh eggs are micrococci which grow poorly, if at all, at the body temperature of the hen (Hadley & Caldwell, 1916; Haines, 1938; Miller & Crawford, 1953). They have been recovered also from ova taken from hens which had been killed and dissected in the laboratory (Harry, 1963*a*). As their recovery was promoted by holding the ova in nutrient broth before plating, it can be assumed that these organs were only lightly contaminated at the time of dissection. Harry (1963*a*) favoured the view that blood-borne organisms are primarily responsible for contamination of the ova. There is ample evidence (Rettger, 1913; May, 1924; Buxton & Gordon, 1947; Gordon & Tucker, 1965) that pathogens such as *Salmonella* spp. pass from the alimentary canal via the blood to the ovaries, but there is no conclusive evidence of such migration by organisms capable of rotting eggs. Thus Miles & Halnan (1937) were unable to promote addling of eggs by feeding or injecting hens with *Proteus melanovogenes*. It seems highly probable, therefore, that rot-producing organisms are primarily of extragenital origin, a surmise that is in agreement with observations that less than 1% of naturally clean eggs rot during prolonged storage (Brooks & Taylor, 1955).

After laying. When eggs were collected aseptically at the time of oviposition, Stuart & McNally (1943) recovered organisms from the

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MICROBIOLOGY OF THE EGG: A REVIEW

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Synopsis

A fairly detailed picture of the course of microbial infection of shell eggs can be prepared from the information available. This indicates that the fate of an egg is determined by the behaviour of organisms which become entangled in the shell membranes. From a commercial viewpoint, it seems that a fuller understanding of this phase of the infection process could be expected to lead to technological innovations whereby microbial deterioration of eggs intended for human consumption could be virtually eliminated. The confinement of effective infection to the shell membrane appears to be due principally to the antimicrobial nature of the white. Many of the proteins of the albumen have been shown to possess, at least *in vitro*, biological properties that might be expected to hinder microbial growth. Conalbumin and lysozyme excepted, there have been no attempts to determine the relative importance, if any, of these substances in the whole egg. It appears that future work should be concerned with the interplay of invading organisms and the antimicrobial defences of the incubating egg. This can be expected to test the biological validity of the currently accepted views of the egg's resistance to infection and may lead to information of value to persons concerned with the marketing of shell eggs or the preparation of egg products.

Introduction

Although microbial deterioration of shell eggs remains a problem to the poultry industry, research in this field is notable for its *ad hoc* nature and relative freedom from critical review. Thus in recent years a marshalling of available evidence has been attempted in four publications only (Haines, 1939; Romanoff & Romanoff, 1949; Brooks & Taylor, 1955; Board, 1966). In the last-mentioned publication the origins of current concepts were sought whereas in the present one emphasis has been given to those features which are of particular importance in establishing methods for safeguarding eggs during distri-

shells of two eggs only. This suggests that the shell of a few eggs are contaminated when passing through the cloaca but that the main contamination occurs after laying. Many investigators (Table 1) have

TABLE 1

Levels of microbial contamination of the shell of the hen's egg

Source of supply	No. eggs examined	Grade and/or treatment of eggs	Number of micro-organisms/shell		Reported by
			Mean	Range	
Farm	36	Handled with gloved hands	$9{\cdot}5 \times 10^3$	*	Rosser (1942)
Batteries	25	*	$2{\cdot}5 \times 10^4$	$2{\cdot}5 \times 10^3 - 8{\cdot}1 \times 10^4$	Harry (1963*b*)
Experimental farm	*	*	$6{\cdot}3 \times 10^4$	$1{\cdot}0 \times 10^4 - 1{\cdot}0 \times 10^6$	Forsythe *et al.* (1953)
Packing station	72	*	$7{\cdot}0 \times 10^4$	*	Rosser (1942)
Farm	36	*	$1{\cdot}0 \times 10^5$	*	Rosser (1942)
Shops and farms	130	*	$1{\cdot}3 \times 10^5$	*	Haines (1938)
Packing station	73	Clean: Grade A	$2{\cdot}2 \times 10^5$	$3{\cdot}0 \times 10^2 - 1{\cdot}0 \times 10^7$	Board *et al.* (1964)
Deep litter	25	*	$3{\cdot}5 \times 10^5$	$6{\cdot}2 \times 10^3 - 2{\cdot}4 \times 10^6$	Harry (1963*b*)
Packing station	77	Lightly soiled: Grade B	$9{\cdot}7 \times 10^5$	$1{\cdot}0 \times 10^3 - 1{\cdot}9 \times 10^7$	Board *et al.* (1964)
Deep litter	96	Clean and lightly soiled	$3{\cdot}1 \times 10^6$	$5{\cdot}0 \times 10^2 - 1{\cdot}0 \times 10^7$	Board & Wilson (1965)

* details not given in original reports.

TABLE 2

Type of micro-organisms recovered from the shell of the hen's egg

Type of organism	Incidence (%) of organisms recovered from the shell of the hen's egg					
	Shops & farms*	Egg breaking plants**			Packing Station ***	
		Clean	Lightly soiled	Heavily soiled	Clean	Lightly soiled	Cracked etc.
Streptococcus	—	8	5	—	—	—	—
Staphylococcus	5	30	—	—	9	5	11
Micrococcus	18	23	20	—	37	52	42
Sarcina	2	20	—	—	—	—	—
Arthrobacter	—	—	—	—	5	13	10
Bacillus	30	—	13	5	—	2·5	—
Pseudomonas	6	—	—	—	22·5	12·5	24
Achromobacter	19	—	—	—	1·5	2	1
Alcaligenes	—	—	—	—	—	2	—
Flavobacterium	3	—	—	—	—	—	—
Cytophaga	—	—	—	—	—	1	—
Coli-aerogenes	5	19	7	5	10·5	7·5	4
Aeromonas	—	—	—	—	1	—	2
Proteus	1	—	20	20	—	—	—
Serratia	—	—	20	50	—	—	—
Moulds	7	—	10	20	—	—	—
Unclassified	—	—	—	—	12†	5†	6†
No. organisms studied	100	NR	NR	NR	130	164	126

* Haines (1938); ** Zagaevsky & Lutikova (1944); *** Board *et al.* (1964)
† aerobic Gram-negative bacteria. NR, numbers not given in report.

been concerned with the number of micro-organisms on the shell and averages in the range $9{\cdot}5 \times 10^3$ to 3100×10^3 organisms/shell have been reported. The extent of contamination appears to be a function of (*a*) the cleanliness of the nest boxes, the heaviest being associated with dirty nesting litter (Harry, 1963*b*), and (*b*) the manner in which the eggs are handled after laying. Thus Rosser (1942) found the level of contamination on the shell of eggs handled with gloved hands was less than when eggs were handled normally.

Gram-positive bacteria are numerically dominant on clean or lightly soiled shells (Table 2) whereas Gram-negative ones can be dominant on badly soiled eggs. These contaminants are almost certainly derived from dust, soil and faeces (Haines, 1939; Zagaevsky & Lutikova, 1944; Board, Ayres, Kraft & Forsythe, 1964).

TABLE 3

Types of organisms recovered from the contents of rotten and tainted eggs

	Occurrence in rotten eggs reported by					Occurrence in tainted eggs reported by Richard & Mohler (1950)
	Haines (1938)	Alford *et al.* (1950)	Florian & Trussell (1957)	Board (1965*b*)	Board & Board (1968)	
Coli-aerogenes	+	+	+	+	+	+
Proteus	+	+	+	+	+	−
Aeromonas	−	−	+	+	+	−
Pseudomonas	+	+	+	+	+	+
Alcaligenes	+	+	+	+	+	+
Achromobacter	+	+	+	+	+	+
Gram-positive bacteria	−	−	−	±	±	±

+, isolated on many occasions; ±, isolated occasionally; −, not isolated.

A mixed infection of Gram-negative bacteria appears to be typical of rotten eggs (Table 3). The nomenclature of these organisms has been discussed elsewhere (Board, 1965*b*) and methods for their rapid characterization and identification have been outlined (Board & Board, 1968). The commonest contaminants of addled eggs are members of the genera *Alcaligenes*, *Achromobacter*, *Pseudomonas*, *Serratia*, *Cloaca*, *Hafnia*, *Citrobacter*, *Proteus* and *Aeromonas*. It is noteworthy that this range of organisms has been obtained from rotten eggs in all continents and that there is no apparent difference in the types recovered from free range eggs (Haines, 1938) or those produced under intensive systems of husbandry (Board, 1965*b*; Board & Board, 1968). Thus their selection can be attributed to factors intrinsic to the egg rather than to features such as housing, temperature of storage and methods of marketing, many of which have changed appreciably in the past two decades. This implies that the rot-producing bacteria, which are in a minority among the contaminants of the shell, have properties that favour their growth in the contents of the egg. These determinants

of spoilage have not been defined in detail. The available evidence indicates that a negative reaction in Gram's staining method, relatively simple nutritional requirements and, with some, an ability to grow at low temperatures may be of particular importance. It is noteworthy that these properties are common also to the organisms recovered from incubating eggs (Harry, 1957; Pathak, Singh & Tangri, 1960; Reid, Macy, Boyd, Kleckner & Schmittle, 1961).

Antimicrobial defence

The shell. The belief (Haines, 1939) that the shell impedes microbial infection of the contents of the egg is based mainly on indirect evidence. That it does so under normal conditions is implicit in the observations that (*a*) less than 1% of the nest clean eggs rot during storage (Brooks & Taylor, 1955), (*b*) the level and incidence of contamination of the contents of eggs having cracked shells are greater than those of undamaged eggs (McNally, 1963; Miller & Crawford, 1953) and (*c*) the rate and incidence of rotting can be increased merely by cracking shells before exposing eggs to rot-producing bacteria (Brown, Baker & Naylor, 1966*c*). The normal shell is perforated with 7,000 to 17,000 pores (Tyler, 1953) the diameters of which are in the range 9 to 35 μ (Romanoff & Romanoff, 1949; Tyler, 1956). There is evidence that when such eggs are breached under farm conditions only ten or so pores provide portals for the entry of microorganisms (Bryant & Sharp, 1934; Orel, 1959). Likewise only a minority of the pores is invaded when eggs are challenged with large numbers of microorganisms under laboratory conditions (Paton & Ayres, 1964; Board & Board, 1967). How such pores differ from those which obstruct microbial penetration is not known but it seems reasonable to assume that debris—the cuticular plug of Simkiss (1961)—within the pore canal plays a part. It has long been suspected (Haines, 1939; Romanoff & Romanoff, 1949; Brooks & Taylor, 1955) that the cuticle, by capping the external orifices of the pores, contributes to the shell's resistance to bacterial infection. Thus it has been shown that rotting in eggs can be promoted merely by rubbing the shell with abrasives before exposing them to rot-producing bacteria (Haines, 1938; Haines & Moran, 1940; Brown, Baker & Naylor, 1966*a*) and that such treatments increase the rate at which the egg loses water during storage (Marshall & Cruickshank, 1938; Tyler, 1945). Recent observations (Simons & Wiertz, 1966) indicate that the cuticle is more complex than had previously been realized and further studies are required before its role in impeding microbial invasion of the shell will be known in detail.

Shell membranes. These appear to offer both mechanical and chemical defence against infection of the albumen.

The concept that the shell membranes behave as bacterial filters arose from the investigations of Haines & Moran (1940). When the

contents of eggs were replaced with suspensions of bacteria, organisms were not recovered from the fluid sucked through the shell but they were present in that coming from shells rid of their membranes. This was confirmed by Walden, Allen & Trussell (1956) and by Garibaldi & Stokes (1958) but they noted that organisms were present in the fluid when suction was not applied until 18 to 24 h after the seeding of the inner surface of the shell membranes. Further evidence that the shell membranes provide only a temporary barrier to bacterial invasion of the albumen came from studies in which shells containing sterile medium were stood in cultures of actively growing bacteria (Garibaldi & Stokes, 1958; Lifshitz, Baker & Naylor, 1964, 1965; Board, 1965*a*). Contamination of the medium within the shell was detected by 6 h or 6 d of incubation. It is noteworthy that these times are of the same magnitude as those recorded by persons who attempted to recover viable organisms from the albumen following inoculation of the air cell. From this site the bacteria must pass through the inner shell membrane, a structure that has a much greater resistance to penetration than either the shell or the outer shell membrane (Lifshitz *et al.*, 1964). In the original work (Miller & Crawford, 1953; Elliott, 1954) organisms were not recovered from the albumen until 4 to 6 d following the seeding of the air cell. Subsequent work has shown that the membrane is quickly breached when large inocula are used (Brooks, 1960; Hartung & Stadelman, 1962; Board, 1964; Board, Henden & Board, 1968) especially when eggs are held at 37°C (Board & Ayres, 1965).

The process which results in the penetration of the shell membranes is not known but it does not appear to be assisted by digestion of these structures by bacterial proteases (Garibaldi & Stokes, 1958; Board, 1965*a*). Membranes taken from rotten eggs have been shown to have lost their ability to retain water (Board, 1962) or a 1% solution of starch (Brown, Baker & Naylor, 1965) and to contain bacteria surrounded by 'zones of hydrolysis' (Brown *et al.*, 1965). The latter authors noted the failure of earlier investigators to implicate bacterial proteases in microbial penetration of the shell yet, without corroborative evidence, they ascribed the 'zones of hydrolysis' to the action of bacterial mucinases or polysaccharidases. Extreme caution has to be exercised when attempting to draw conclusions from observations made on material derived from rotten eggs. It will be seen later that organisms contained in the shell membrane during the primary phase of multiplication appear to be physiologically less active than those present within the egg at the time when rotting of the contents is occurring.

The concept that the shell membranes provide the egg with a chemical means of defence rested initially on evidence derived from *in vitro* studies. Korotkova (1957) detected lysozyme in these membranes and considered that this played a part in defending the developing embryo against Gram-positive bacteria. It is unlikely to play other than a marginal part in eggs intended for human consumption (Fig. 1).

The belief that the membranes may also destroy Gram-negative organisms stemmed from observations (Stuart & McNally, 1943) that there was a rapid die-off when such organisms were held in a saline suspension of shell membranes. Subsequent investigations have shown that, providing a non-toxic suspending medium is used (Elliott & Brant, 1957), good growth of the common contaminants of rotten eggs occurs with intact (Board, 1965*a*) or comminuted membranes (Stokes & Osborne, 1956; Garibaldi & Stokes, 1958).

As will be seen later, there is a lag of 10 to 20 d following bacterial penetration of the shells of newly laid eggs and the occurrence of large numbers of organisms in the albumen. The possibility that this may be caused by antimicrobial substances within the shell membranes *in situ* has been tested with the following technique (Fig. 1). Newly laid eggs were candled and those of poor internal quality discarded. The shell

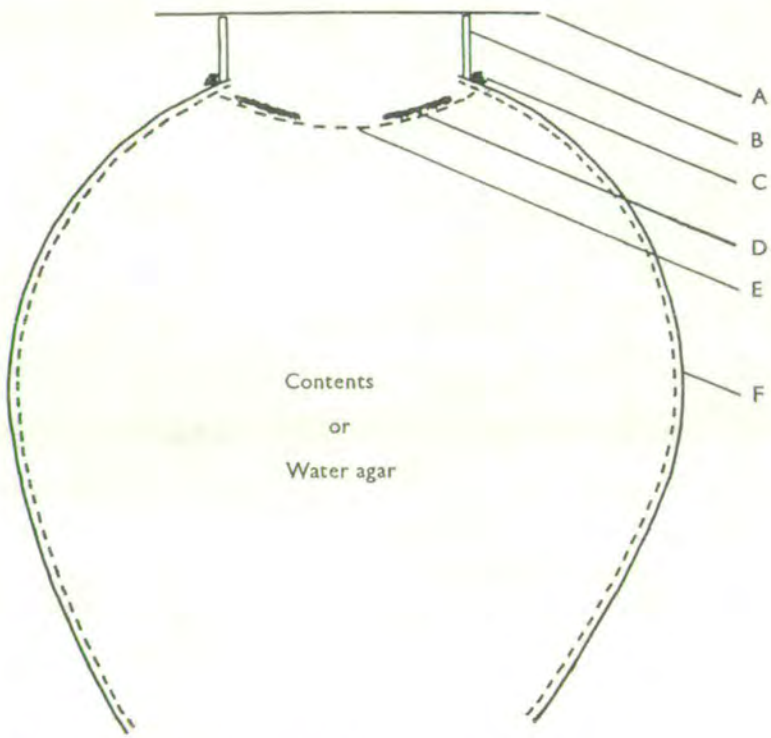

FIG. 1. Method used to study behaviour of organisms resting on the shell membranes *in situ*.

A . . . . coverslip.
B . . . . glass ring (height, 15 mm; internal diameter, 7 mm).
C . . . . paraffin wax.
D . . . . filter paper disc (diameter, 6 mm).
E . . . . inner shell membrane of air cell.
F . . . . shell.

above the air cell was swabbed with 70% ethanol. A carborundum disc was used to cut the shell above the air cell but care was taken to ensure that the underlying membrane was not severed. A glass ring (internal diameter, 15 mm; height, 7 mm) was cemented (paraffin wax) to the shell surrounding the cut. The inside of the ring was wiped with a burning pledget of cotton wool containing ethanol. With the egg resting on its side, the piece of severed shell together with the attached membrane was removed and the glass ring immediately covered with a cover-slip coated with sterile petroleum jelly. Some of the eggs had their contents removed and, after flushing with sterile water, the shells were filled with agar (2%, w/v) dissolved in distilled water. Discs of filter

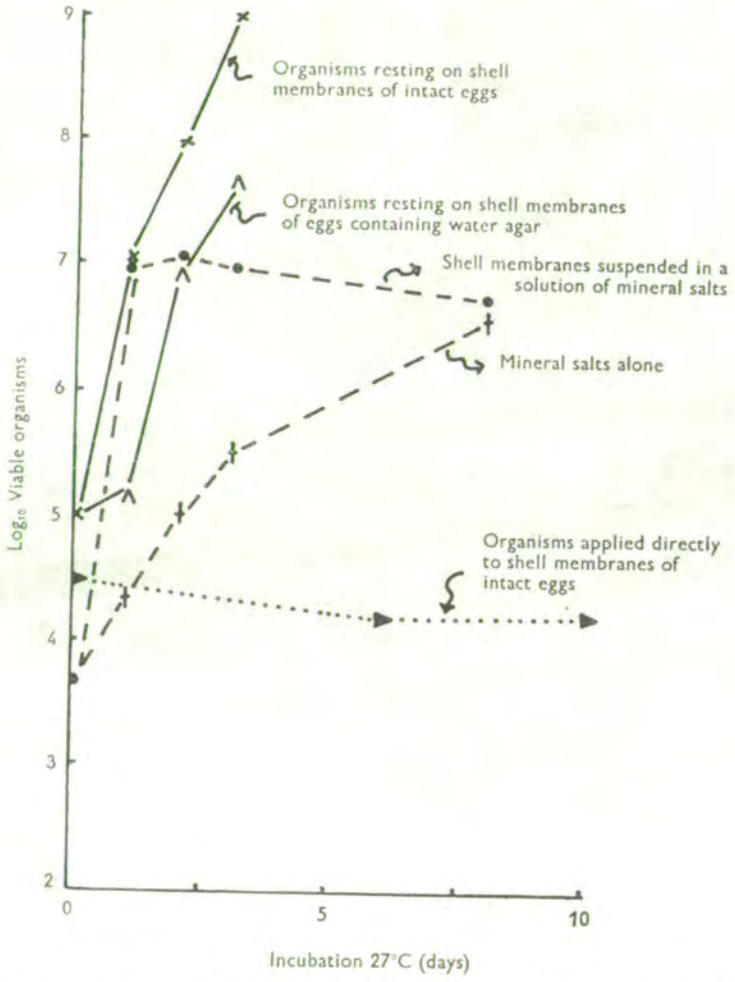

FIG. 2. The growth of *Cloaca* G75/2 on shell membranes *in situ* compared with that on membranes contained in a solution of mineral salts

paper (Whatman No. 1, diameter 6 mm) moistened in a dilute suspension of the test organism were placed on the outer surface of the air cell, which would accommodate 4 discs. The eggs were incubated at 27°C and, at frequent intervals, one of the discs removed. This was comminuted by shaking in a screw-capped glass vial containing 6 glass beads and 2 ml of water. Serial decimal dilutions were prepared in quarter-strength Ringer's solution and 0·02 ml of appropriate dilutions placed on the surface of nutrient agar (Oxoid, London). The colonies were counted after 3 d at 27°C.

The results (Fig. 2) obtained with this technique are exemplified by those obtained with *Cloaca* G75/2. The fastest and most extensive growth occurred with organisms contained in filter paper resting on shell membranes *in situ*. The populations developing in whole eggs were invariably larger than those in egg containing water agar. The growth characteristics of G75/2 also added weight to an interpretation that growth on the surface of the shell membranes is influenced by substances in the underlying albumen. Thus it had been noted that abundant viscous material was formed when this organism grew in a range of commonly used laboratory media. This material was formed only in whole eggs: its presence caused the filter paper to adhere firmly to the shell membrane. In common with results discussed elsewhere (Board, 1964), no growth occurred in the few days following the application of an aqueous suspension of the organism to the shell membranes of entire eggs. When membranes were suspended in a solution of mineral salts, growth was at a rate of the same magnitude as that occurring in filter paper supported by the membranes *in situ*. Results similar to these were obtained with (the bracketed figures refer to the number of strains tested): *Pseudomonas fluorescens* (2), *Pseudomonas maltophilia* (2), *Aeromonas liquefaciens* (1), *Proteus vulgaris* (2) and *Alcaligenes faecalis* (1). This evidence indicates that the shell membranes themselves do not contain antimicrobial substances in significant amounts but that *in situ* the environment within them is influenced by substances in the underlying albumen. The available evidence implicates conalbumin. Thus the growth of rot-producing bacteria in the membranes is promoted by the addition of trace amounts of iron to the infected membrane (Board *et al.*, 1968) or by the saturation of the iron-chelating potential of the conalbumin in the albumen (Board, 1964). That this ligand can exert an influence through semipermeable membranes was shown by Feeney & Nagy (1952).

The albumen. The biological properties of the proteins of the albumen are given in Table 4. These data have provided the basis for the currently accepted concept of the antimicrobial defence of the albumen, namely that it is a medium unsuitable for microbial growth. The events leading to an acceptance of this view have been given elsewhere (Board, 1966). At this time it will suffice to consider in detail only those components which appear to have a principal role in the egg's defence.

TABLE 4

The biological properties of components of the albumen of the hen's egg

Component		Action	Investigator
Lysozyme		Lysis of cell walls of certain bacteria	Laschtschenko (1909)
		Flocculation of bacterial cells	Fleming (1922)
		Hydrolysis of β-1-4- glycosidic bonds	Freidberger & Hoder (1932); Berger & Weiser (1957)
Conalbumin		Chelation of iron, zinc and copper	Schade & Caroline (1944); Alderton, Ward & Fevold (1946)
Ovomucoid		Inhibition of trypsin	Balls & Swenson (1934); Lineweaver & Murray (1947)
Avidin		Combination with biotin	Eakin, Snell & Williams (1940); Woolley & Longsworth (1942); Baumgartner (1957)
Riboflavin		Chelation of cations	Feeney & Nagy (1952)
Uncharacterized proteins	A	Inhibition of trypsin and chemotrypsin	Rhodes, Bennett & Feeney (1960)
	B	Inhibition of fungal protease	Masushima (1958)
	C	Combination with riboflavin	Rhodes, Bennett & Feeney (1959)
	D	Combination with vitamin B_6	Evans, Butts & Davidson (1951)
	E	Chelation of calcium	Abels (1936)

Laschtschenko (1909) observed lysis of *Bacillus subtilis* seeded into albumen and noted that this did not occur with egg white which had been held at 65 to 70°C for 30 min. The enzymic nature of this phenomenon was confirmed by Fleming (1922) and he proposed the name lysozyme for the lytic agent. Today it is more correctly referred to as β-N-acetyl-hexosaminidase and, together with related enzymes from other sources, classified as a muramidase (Salton, 1964). Until ten years ago there was only a slow acquisition of data concerning the action of this enzyme (Thompson, 1940, 1941; Salton, 1957) but it was established that under normal conditions Gram-positive bacteria are on the whole more sensitive to lysozyme than Gram-negative organisms. An unquestioned acceptance of this has led the majority of egg microbiologists to assume that lysozyme is the primary reason for the absence of Gram-positive bacteria in rotten eggs.

An understanding of the action of lysozyme has come from studies concerned with the chemical composition and organization of the bacterial cell wall. The cytoplasmic membrane of all true bacteria is encased in a rigid bag—a macromolecular net—to which various names have been given: the basal structure (Work, 1957), murein layer (Martin, 1963) or murein sacculus (Weidel & Pelzer, 1964). This can be considered to be the morphological and functional equivalent of an egg shell. It endows the cell with geometry and protection against

damage that might otherwise be caused by changes in osmotic pressure. The murein sacculus consists of muropeptides (Weidel & Pelzer, 1964) —known also as mucopeptides (Perkins & Rogers, 1959)—with the composition shown in Fig. 3. They are linked together in part by petide and in part by β-1-4 and β-1-6 glycosidic bonds. It is the β-1-4 bonds that are attacked by lysozyme (Berger & Weiser, 1957; Salton & Ghuysen, 1960).

The cell wall of the organism, *Micrococcus lysodeikticus*, used by Fleming (1922) is formed mainly from muropeptides: 80 to 90% on a weight basis (Perkins & Rodgers, 1959). It is easily depolymerized by lysozyme, presumably by virtue of the fact that the majority of β-1-4 glycosidic bonds are exposed. A similar situation is found in *Bacillus megaterium* and lysis of its cell wall causes the rod-shaped (Fig. 3) organism to assume a spherical form (a protoplast) which, if not suspended in an isotonic solution, is quickly ruptured by plasmolysis (Weibull, 1953*a*, *b*). This is presumably what Laschtschenko (1909) observed when he added *Bacillus subtilis* to egg white. The cell wall of the latter organism contains appreciable amounts of teichoic acids (Armstrong, Baddiley, Buchanan, Carrs & Greenberg, 1958; Baddiley, Buchanan & Carrs, 1958) but these do not prevent lysozyme from reaching its substrate. With other Gram-positive bacteria, however, resistance to lysozyme appears to be due to cell-wall accessories masking the muropeptides. Thus with certain strains of *Listeria monocytogenes*, the cells have to be treated with a lipase before they can be broken down by lysozyme (Ghosh & Murray, 1967). It would be naive to expect organisms with the last-mentioned properties to be invariably absent from the microflora on the shell of the hen's egg. Thus it would appear that the occurrence of a predominantly Gram-negative flora in rotten eggs is due only in part to the action of lysozyme.

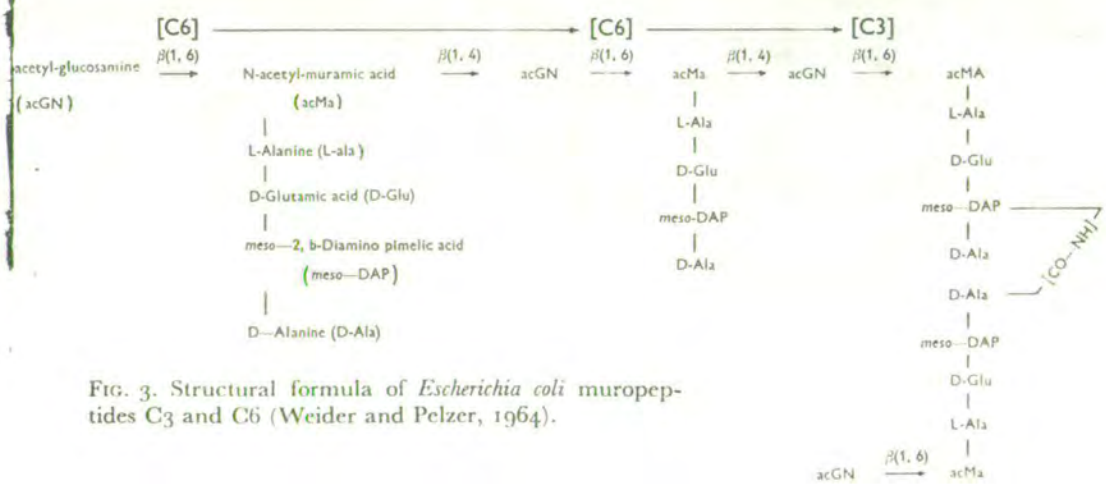

FIG. 3. Structural formula of *Escherichia coli* muropeptides C3 and C6 (Weider and Pelzer, 1964).

The cell walls of Gram-negative bacteria are structurally more complex than those of Gram-positive ones (Fig. 4). The murein sacculus, which constitutes about 5% of the cell wall (Rogers, 1965), is studded with protein aggregates and overlaid with lipoprotein and lipopolysaccharide (Kellenberger & Ryter, 1958; Frank & Martin, 1960; Martin, 1963). How the outer layers are held to the basal structure is not known in detail but the available evidence implicates a combination of ionic and hydrogen bonds (Gray & Wilkinson, 1965*a*, *b*; Rogers, 1965; Wolin, 1966; Birdsell & Cota-Robles, 1967; Asbell & Eagon, 1966; Nermut & Murray, 1967; Weinbaum, Rich & Fischman, 1967).

In spite of the location of the murein sacculus, Gram-negative bacteria can be rendered susceptible to the action of lysozyme. Formerly this was achieved by ancillary procedures applied prior to or along with exposure to the enzyme. Thus incubation under alkaline conditions (Zinder & Arndt, 1956) or freezing and thawing (Kohn, 1960) induces lysozyme-sensitivity in many Gram-negative organisms. Similarly the incubation of cells in an alkaline solution of ethylenediamine-tetraacetic acid (EDTA) and lysozyme causes lysis of Gram-negative organisms as witnessed by the decline in opacity of cell suspensions (Repaske, 1956, 1958; Vos, 1964). Recently it has been shown that very young cells of *Escherichia coli* are sensitive to lysozyme under alkaline conditions (Birdsell & Cota-Robles, 1967). Similarly it has been demonstrated that lysozyme alone or in combination with EDTA does not invariably cause the rod-shaped organisms to assume the spherical (spheroplast) form; they retain their normal shape but are extremely sensitive to slight changes in osmotic pressure (Vos, 1964; Asbell & Eagon, 1966). This implies that muropeptides are not solely responsible for endowing cells with geometry and evidence is at hand (Asbell & Eagon, 1966; Carson & Eagon, 1966; Weinbaum & Markman, 1966) which suggests that lipopolysaccharides are concerned with strengthening the cell envelopes. In the light of these data it would appear to be unwise to consider that lysozyme has no importance in defending eggs against infection by Gram-negative bacteria. A feature of the treatments needed for the sensitization of such organisms to the action of this enzyme is an agent (EDTA) capable of chelating divalent cations, especially Ca^{++}, and an alkaline reaction. It is worthy of comment, therefore, that the white of an egg contains the chelating agents albumen (Abels, 1936), conalbumin (Schade & Caroline, 1944; Alderton, Ward & Fevold, 1946) and riboflavine (Feeney & Nagy, 1952) and that its pH becomes steady at 9·6 within 3 d of the egg being laid (Healey & Peter, 1925). Thus an assessment of the true role of lysozyme in the defence of the hen's egg must await further studies.

Schade & Caroline (1944) noted inhibition of microbial growth in nutrient broth supplemented with egg white when the mixture was adjusted to pH 7·4 or more but not when it was adjusted to pH 5·8 or less. Of ten vitamins and thirty-one elements tested, iron alone over-

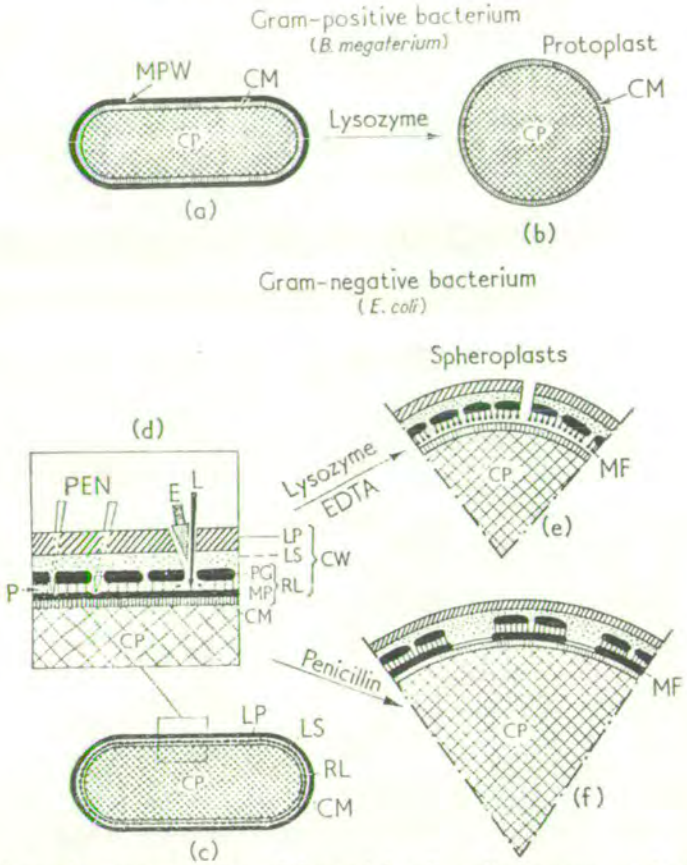

FIG. 4. Schematic representation of bacterial cell wall structure and of changes occurring in cell walls during treatment with lysozyme and penicillin.

(a) Gram-positive bacterium in which the protoplast within the cytoplasmic membrane (CM) is surrounded by a presumably pure mucopolymer cell wall (MPW). Lysozyme dissolves the cell wall and liberates the naked protoplast (b). (Data of Weibull, 1953*a*; Thorsson & Weibull, 1958.)

(c), (d) Gram-negative bacterium with complex triple-layered cell wall. Separation of the layers can be achieved by solvent extraction and by treatment with proteolytic enzyme (P). Both lysozyme (L), aided by EDTA (E) and penicillin induce depolymerization of the rigid mucopolymer (MP), although probably to a different degree and in a different way. Only small mucopolymer fragments (MF), which are covalently linked to other cell wall components, remain in the wall after lysozyme treatment (e). Penicillin spheroplasts and the related L-forms may retain larger mucopolymer fragments of a modified, non-rigid mucopolymer in their stretched cell walls which surround greatly increased protoplasts (f). (Data of Kellenberger & Ryter, 1958; Hofschneider, 1960*c*; Weidel *et al.*, 1960; Frank & Martin, 1960; Kandler *et al.*, 1958*a*; Martin & Frank, 1962*b*.)

LP, lipoprotein layer; LS, lipopolysaccharide layer; RL, rigid layer; PG, protein granula; MP, mucopolymer; CM, cytoplasmic membrane; CP, cytoplasm; MF, mucopolymer fragments; PEN, penicillin; L, lysozyme; E, ethylene diamine tetra-acetic acid; P, proteolytic enzyme.

[Fig. 4 and caption reproduced from Martin 1963 by permission of the author and the publishers, Academic Press (London) Ltd.]

came the inhibition. The iron-binding, inhibitory substance was isolated from egg white and identified with conalbumin (Alderton *et al.*, 1946). This organic ligand differs from the β_1 iron-binding serum albumin, transferrin or siderophilin, only in the composition of its carbohydrate prosthetic group (Williams, 1962). It accounts for 10% of the total egg-white solids (Longsworth, Cannan & MacInnes, 1940); it is uniformly distributed throughout the white (Feeney, Ducay, Silva & MacDonnell, 1952); its sequestering power is not detectably reduced by short terms of storage of eggs (Feeney *et al.*, 1952) and there is no evidence that its chelating potential is satisfied to any significant extent by iron diffusing from the yolks of stored eggs (Schaible, Bandemer & Davidson, 1946).

When purified preparations of conalbumin or siderophilin have been used to inhibit microbial growth, it has been established that they must be present in stoichiometric excess of the iron found in the medium by chemical analysis (Fraenkel-Conrat & Feeney, 1950; Feeney & Nagy, 1952; Schade, 1958, 1961). Inhibition expresses itself by an increase in the lag phase of growth, a decreased rate of multiplication once growth has begun and, when large amounts of conalbumin are present in a medium, a marked reduction in the size of the final populations (Fraenkel-Conrat & Feeney, 1950; Feeney & Nagy, 1952; Schade, 1963; Theodore & Schade, 1965*a*). These workers noted also that different organisms show different degrees of inhibition—micrococci are more sensitive than *Bacillus* spp. and the latter are more sensitive than Gram-negative bacteria—and that an increase in the alkalinity of a medium increased further bacterial inhibition. When a medium is rendered iron-deficient through the addition of these organic ligands, the production of pigment by pseudomonads is enhanced (Feeney & Nagy, 1952) but, as would be expected, the respiratory efficiency of *Staphylococcus* spp., especially in the utilization of intermediates of the Krebs cycle, is greatly impaired (Schade, 1958, 1963; Theodore & Schade, 1965*b*).

With organisms derived from rotten eggs, Garibaldi (1960) noted that good growth occurred in albumen *in vitro* when the pH was adjusted to a neutral reaction or when the chelating potential of conalbumin was satisfied by the addition of iron. This was confirmed by Brooks (1960) and essentially similar results have been obtained in experiments with whole eggs (Board, 1964; Board *et al.*, 1968). The latter authors showed that extensive bacterial multiplication occurred when iron was added either to the shell membranes or the albumen. Natural or artificial contamination of water with iron salts also results in a high incidence and rate of rotting in washed eggs (Garibaldi & Bayne, 1960, 1962*a*, *b*; Brant & Starr, 1962). Thus the available evidence warrants the conclusion that the chelation of iron by conalbumin is the primary factor in preventing the growth of lysozyme-insensitive organisms in the hen's egg.

That it is not the sole factor is evident in the results of Brooks (1960). He could not obtain in albumen supplemented with iron rates of bacterial multiplication of the same order as those in a favourable medium. This led him to suggest that shortage of available nitrogenous substances might be important. This aspect was considered in detail by Haines (1939) but its importance is still conjectural. This is the case, also, with the components other than lysozyme and conalbumin listed in Table 4. It is felt that a detailed understanding of their role, if any, in the antimicrobial defence of the albumen must await studies in which particular importance is attached to the true biological role of this defence. In other words, what part does it play in defending the embryo in the first few days of incubation?

Course of infection

There are three major stages leading up to the rotting of an egg (Gillespie and Scott, 1950), namely (*a*) bacterial penetration of the shell, (*b*) colonization of the shell membranes and (*c*) infection of the albumen.

Penetration of the shell. It was noted above that the mechanisms whereby microorganisms penetrate the shell are not known. In this section attention will be given to those practices that are likely to promote microbial invasion of the shell under commercial conditions.

Ferdinandov (1944) claimed that the shell is susceptible to bacterial penetration within a short time of laying. He attributed this to the yolk and white contracting on cooling thereby drawing organisms into pores, the canals of which were not effectively blocked because the cuticle and pore debris were still moist. This allows an interpretation of the observations (Lorenz *et al.*, 1952; Dr J. Patton, personal communication) that a high incidence of rotting occurs during the storage of eggs gathered from nests in which the litter was contaminated deliberately with large numbers of rot-producing bacteria. This would appear, however, to be an unusual mode of infection under normal conditions since it is well established (Gillespie, Scott & Vickery, 1950*a*; Brooks & Taylor, 1955) that less than 1% of nest clean eggs rot during prolonged storage.

Water appears to be an essential agent for bacterial penetration of the shell; it is drawn in by capillary attraction when the egg and water are at the same temperature, or it is sucked in when a warm egg contracts on cooling. In either case microorganisms on the shell are carried through the ten or so pores (Bryant & Sharp, 1934; Orel, 1959) which, for reasons unknown, are easily breached. When capillarity is responsible for microbial invasion of an untreated shell, organisms can be recovered from the inner surface of the shell membranes (Stuart & McNally, 1943; Wilson, 1945; Buxton & Gordon, 1947; Lancaster & Crabb, 1953; Rizk, Ayres & Kraft, 1966*a*) and upwards of 18 to 28% of the eggs rot during storage (Haines & Moran, 1940; Gillespie, Scott

& Vickery, 1950*a*). A higher incidence of contamination of the shell membranes (Gordon & Tucker, 1954; Brown *et al.*, 1966*a*) and a significantly greater incidence of rotting (Haines, 1938; Haines & Moran, 1940; Fromm & Monroe, 1960; Brown *et al.*, 1966*a*) occur when the shells are rubbed or treated with chemicals (HCl, EDTA) which attack $CaCO_3$. An interpretation that such treatments unblock some of the pores is supported by the observation that they increase the shell's permeability to alcoholic solutions of methylene blue (Fromm & Monroe, 1960; Brown *et al.*, 1966*a*).

Heavy contamination of the shell membranes (Bean & MacLaury, 1959; Brown *et al.*, 1966*a*; Rizk *et al.*, 1966*a*) and a high incidence of rotting (Haines & Moran, 1940; Brown *et al.*, 1966*a*) occur in eggs which are warm when dipped in a cold suspension of rot-producing bacteria. Because of its importance in washing eggs intended for market, this phenomenon has been examined in detail and the following factors play an important part: (*a*) the temperature differential between the egg and the bacterial suspension, the incidence of rotting being directly proportional to the difference in temperature in the range 6 to 21°C (Lorenz *et al.*, 1952; Brant & Starr, 1962); (*b*) the number of organisms in the suspension (Lorenz *et al.*, 1952; Stokes, Osborne & Bayne, 1956; Brant & Starr, 1962; Hartung & Stadelman, 1963); (*c*) the period of immersion (Brant & Starr, 1962; Hartung & Stadelman, 1962); (*d*) the thickness of the shell, thin shells offering less resistance than thick ones (Orel, 1959); and (*e*) the treatment of the shell before immersion, the incidence of rotting being increased when the shells are rubbed with abrasives (Brown *et al.*, 1966*a*). In the light of these findings it is not surprising that many of the older types of egg washing machine have been associated with high incidence of rotting in stored eggs (Gillespie, Salton & Scott, 1950; Gillespie, Scott & Vickery, 1950*b*; Trussell, Fulton & Cameron, 1955; Trussell, Triggs & Greer, 1955; Knowles, 1957*a*, *b*). In a recent review concerned with the design of egg washers (Brant, Starr & Hamann, 1966) the authors described a machine which under commercial conditions proved to be an effective cleaner without contributing significantly to spoilage in stored eggs. An important feature of this machine appears to be maintenance of the temperature of the wash water at 11 deg C above that of the egg.

Bacterial multiplication does not appear to occur in the shells of eggs held under normal conditions of storage (Haines, 1938; Forsythe, Ayres & Radlo, 1953; Board *et al.*, 1964). This has been attributed to a level of available water insufficient for the growth of commonly occurring microorganisms (Sharp & Stewart, 1936; Gillespie & Scott, 1950). Under experimental conditions it has been noted that bacteria die when placed on shells provided that the latter are not dirty and the eggs are not held under humid or chilled conditions (Wolk *et al.*, 1950; Lancaster & Crabb, 1953; Cotterill & Gardner, 1957; Magwood, 1964;

Rizk, Ayres & Kraft, 1966*b*). Thus the egg handler need not be concerned with an increase in the level of contamination of the shell during the distribution of eggs but he must be cautious when attempting to reduce it by chemical means.

It has been noted above that even the most rigorously controlled procedures do not ensure the sterilization of the shells of all eggs. Similarly under farm conditions the presence of bactericides in wash water achieves nothing more than a reduction in the level of contamination of the shell (Winter, Burhart & Wettling, 1952; Winter, Burkart, Clements & MacDonald, 1955). Moreover their use does not guarantee freedom from spoilage during the storage of washed eggs (Gillespie, Salton & Scott, 1950; Sauter, Petersen & Lampman, 1962; Sauter, 1966). Under such conditions viable organisms penetrate to the shell membranes where they are protected from inimical agents through the latter's combination with proteinaceous material (Cotterill & Hartman, 1956; Elliott & Romoser, 1957; Schmidt & Stadelman, 1957; Bean & MacLaury, 1959; Rizk *et al.*, 1966*a*). Thus disinfectants cannot be expected to correct faults arising from an inappropriate choice of cleaning methods.

Deterioration due to microbial action is of rare occurrence in eggs which have been held temporarily at temperatures of 57·5 to 70°C (Goresline, Moser & Hayes, 1950; Funk, 1943, 1948; Romanoff & Romanoff, 1944; Murphy & Sutton, 1947; Salton, Scott & Vickery, 1951; Funk, Forward & Lorah, 1954; Scott & Vickery, 1954; Knowles, 1956). This has led to the tacit assumption that organisms on and in the shell are destroyed during 'shell pasteurization'. Could it not be that they are not killed but due to physiological damage their growth is prevented by the unfavourable environments of the shell membranes? There is abundant evidence to support such an interpretation (Nelson, 1943; Heater & van der Zant, 1957). It is surprising, also, that this method has not enjoyed a wide application in commerce. Future studies could well be expected to give a clearer understanding of this facet of egg microbiology and suggest means whereby 'shell pasteurization' could be achieved as an integral part of cleaning eggs in the machines described by Brant *et al.* (1966).

Colonization of the shell membranes. It can be presumed that commercial conditions favouring microbial invasion of the shell will cause the shell membranes to be infected with a random selection of those organisms which are normally resident on the shell (Table 2). As the infection will be dominated by Gram-positive bacteria it is surprising that egg microbiologists have shown no concern for the fate of these organisms. This aspect has been examined (Board & Board, unpublished work) and the results summarized in Fig. 5.

Shells of badly soiled eggs were ground to a paste in sterile water. A sample of this was mixed with lysozyme (5 μg/ml paste) and, after incubation for 10 min, it was diluted and 1 ml of appropriate dilutions

spread over nutrient agar. Another sample of paste was diluted and spread on nutrient agar. The viable counts obtained with the former ($5{\cdot}0 \times 10^4$ organisms/ml paste) did not differ significantly from those of the latter ($8{\cdot}0 \times 10^4$/ml), thus indicating that few lysozyme-sensitive organisms were present on the shell. Known volumes (0·1 ml) of the paste were injected into the air cell of newly laid eggs. These, with their air cells lowermost, were held at 4 or 27°C and the size of the populations in the inner membrane of the air cell was determined by the methods given elsewhere (Board, 1964). In addition to the enumeration of viable organisms, films were prepared from a random selection of the colonies developing on nutrient agar and stained by Gram's method so that gross changes in the composition of the populations could be detected.

Apart from a slight lag in the first 24 h incubation at 4°C (Fig. 5), there was a marked increase in the populations present in the inner

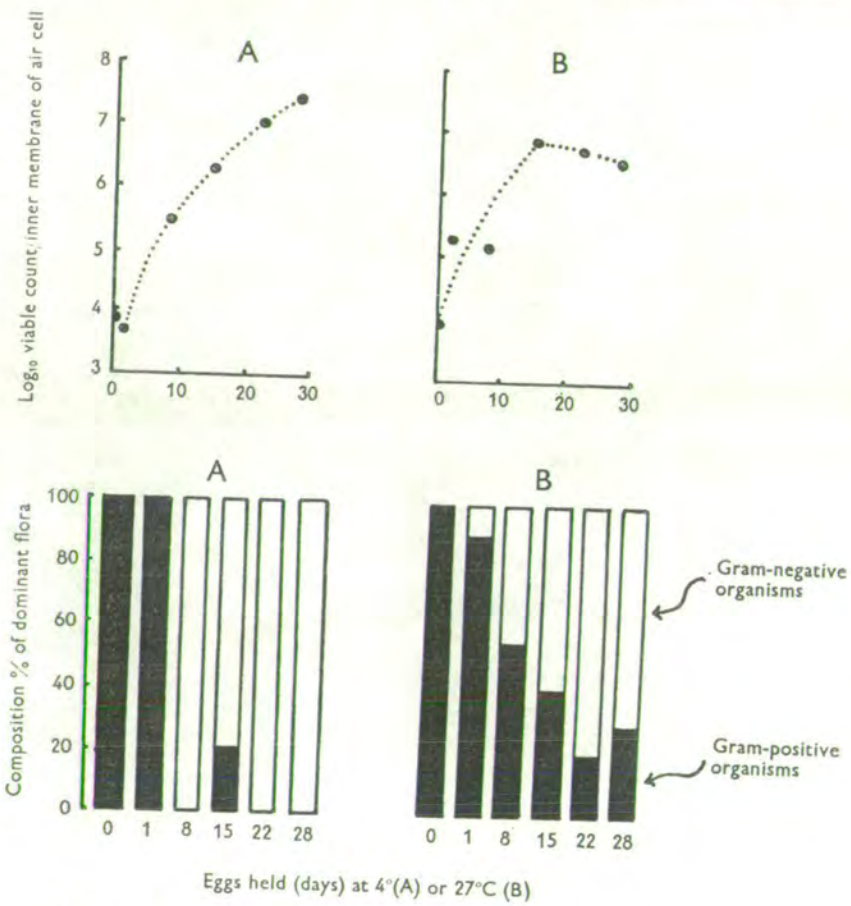

FIG. 5. Growth of organisms in shell membranes infected with a paste prepared from the shells of badly soiled eggs.

membrane of the air cell. It is suspected that under the conditions of these experiments bacterial multiplication was promoted by iron derived from the soiled shells. Gram-positive bacteria were numerically dominant in eggs held for 2 to 3 d at 4 or 27°C. Thereafter, Gram-negative organisms became dominant particularly in eggs held at 4°C. These results indicate that a process of selection, determined by rate of growth rather than by sensitivity to lysozyme, is the main feature of the early stages of infection of the shell membranes.

In eggs contaminated under commercial conditions and held at 15 to 30°C, the infection remains confined to the shell membranes for upwards of 15 to 20 d (Zagaevsky & Lutikova, 1944; Gillespie & Scott, 1950; Bigland & Papas, 1953; Miller & Crawford, 1953; Elliott, 1954; Stokes *et al.*, 1956; Orel, 1959; Fromm & Monroe, 1960; Garibaldi & Bayne, 1960). It is noteworthy that this lag is not influenced appreciably by the number of organisms used to challenge the eggs but it is reduced slightly when the challenge is directed at the broad rather than the pointed end of the shell (Lorenz *et al.*, 1952). This confinement has been attributed to the shell membranes providing a chemical and/or physical barrier to infection (Brooks & Taylor, 1955; Kraft, Elliott & Brant, 1958; Hartung & Stadelman, 1962) but recent evidence (Brooks, 1960; Board, 1964) suggests that it is due to the antimicrobial nature of the albumen. Such an interpretation is supported by the observations of Brown, Baker & Naylor (1966*b*). They noted that eggs inoculated directly into the albumen rotted at the same rate as those the shell membranes of which had been seeded with pseudomonads.

When commonly occurring contaminants of rotten eggs are introduced to the inner membrane of the air cell and the eggs held at 20 to 30°C, a primary phase of multiplication occurs in the first 2 to 4 d of incubation (Brooks, 1960; Board, 1964). When small inocula are used, this results in a relatively small increase in the size of the populations in the inner membrane of the air cell, but when large inocula are used the albumen too is invaded but there is no multiplication. The limitation of this initial phase of multiplication is due to the action of conalbumin (Board, 1964).

The period of decline which follows the primary phase of multiplication results presumably from the death of organisms in the membranes or their migration to the albumen. Later a secondary phase of multiplication occurs. Its induction has been attributed to a spontaneous change in the properties of the shell membranes (Brooks, 1960; Elliott & Brant, 1957; Hartung & Stadelman, 1963). On the other hand, Sharp & Whitaker (1927) considered that multiplication of the organisms which invade the albumen does not occur until they have made contact with the yolk. This appears to be the case with eggs held at 10°C or less (Board & Ayres, 1965) but not in those held at 10 to 37°C. In the latter case, renewed multiplication of the organisms in the shell membranes is associated with a union of the yolk and infected mem-

branes (Board, 1964; Board & Ayres, 1965). This suggests that the duration of the lag phase noted above is determined in part by the rate at which the yolk moves towards the membranes and implies that the internal quality of eggs should be considered to play some role in the antimicrobial defence.

When eggs infected with Gram-negative bacteria are held at 10°C or less, there are not two distinct phases of multiplication (Board & Ayres, 1965; Board *et al.*, 1968). Slow multiplication of the organisms in the shell membranes occurs throughout incubation and this is associated with a slow but progressive increase in the level of contamination of the albumen. The onset of rotting is not associated with a union of the yolk and shell membranes and it would appear that it results from the growth of organisms on the surface of the yolk (Sharp & Whitaker, 1927).

Yet another pattern of events is seen when eggs are inoculated with organisms suspended in a dilute solution of $FeSO_4.7H_2O$ (Board, 1964; Board *et al.*, 1968). Organisms retained by the shell membranes multiply rapidly and the albumen becomes heavily contaminated. The latter organisms appear to remain quiescent until they reach the yolk, at which time rotting becomes evident in eggs containing chromogenic and/or proteolytic organisms.

Growth in the albumen. The evidence reviewed above indicates that a distinction should be drawn between albumen containing resting or moribund organisms and that containing dividing organisms. From the material reviewed to date, it is apparent that no explanation founded on direct evidence can be offered to account for the quiescence of the initial contaminants of the albumen although it may well be due to conalbumin either alone or in conjunction with other antimicrobial substances of the albumen (Table 4). During this state they appear incapable also of utilizing the most readily available energy source, namely glucose (Board, 1964; Board *et al.*, 1968; Dr D. J. Stewart, personal communication). Similarly, the mechanisms leading to a switch from the resting to the dividing state are fairly well documented but, again, there are no details concerning the chemistry of this change; can it be that the yolk supplies the organisms with iron?

Conclusions

The terms of ecology are wholly appropriate for a detailed description of the course of microbial infection of eggs. They have the additional merit of providing a simple means of indexing extant knowledge and giving emphasis to gaps therein. Thus the term *association* can be used for the characteristic flora of rotten eggs (Table 3) and its genesis under practical conditions can be considered to be determined by (*a*) the initial *infection* of the shell, (*b*) conditions pertaining in the egg (*intrinsic* factors), (*c*) the storage environment (*extrinsic* factors) and

(*d*) the properties of organisms making up the association (*implicit* factors).

Infection. The shells of eggs are contaminated with organisms from ubiquitous depots, dust, soil and faeces. The composition of this flora can be expected to be determined by geographic and climatic factors and its size by the form and quality of husbandry. As yet there is no convincing evidence that any of these influences the actual process of infection: Gram-negative bacteria are always present on the shell and some of them will cause deterioration if eggs are improperly handled.

Intrinsic factors. This refers to the antimicrobial defence. Under commercial conditions no guarantee can be given that the shells of *all* eggs will not be invaded by microorganisms. Likewise chemical means cannot be relied upon to rid the shell and underlying membranes of microorganisms. 'Shell pasteurisation' has been shown to be an effective method under closely controlled conditions but, for reasons unknown, it has not enjoyed a wide application in industry. The available evidence emphasizes the cardinal importance of the shell membranes in the infection process. They ameliorate the antimicrobial defence of the albumen and provide a nidus for the ultimate infection of the yolk and white of eggs held in the range 1° to 30°C. Of the antimicrobial components of the albumen, conalbumin in association with the alkaline reaction and lysozyme appears to be of importance. The actual quality of the albumen may also play a part through determining the rate at which organisms migrate towards the yolk.

Extrinsic factors. In this context, promotion of spoilage of eggs washed in water containing iron provides an example of a detrimental factor. In general, however, extrinsic factors appear to offer the best methods for controlling or preventing microbial deterioration of eggs. Temperature has long been recognized as an important means of controlling egg quality but, due to irreparable damage caused by holding eggs below 0°C (Hale, 1950), it cannot entirely prevent deterioration arising from microbial action. Thus in the range 1 to 10°C, spoilage is only retarded through a reduction in the rate of microbial multiplication. In this range, also, psychrophilic organisms in the shell membranes grow at the expense of mesophilic ones (Stokes *et al.*, 1956; Rizk *et al.*, 1966*b*) whereas the reverse is the case in eggs held at room temperature (Alford, Holmes, Scott & Vickery, 1950; Frazier, 1967). It would appear, therefore, that control of the temperature of storage together with a modification of the gaseous environment surrounding the eggs may provide the most satisfactory means of arresting microbial deterioration. There is abundant evidence concerning the properties of transparent films and their role in maintaining internal quality (Cotterill & Gardner, 1957; Fletcher, Orr, Snyder & Nicholson, 1959; Davis & Beeckler, 1962) and hatchability of eggs (Warren, Roff & Long, 1965; Proudfoot, 1964, 1965, 1966*a*, *b*; Bowman, 1966). As yet, however, there is no information on the actual composition of gas

TABLE 5

Changes in eggs produced in 42 days at 27°C by different bacterial species and a presumptive correlation of the salient features of the rots with the metabolic attributes of the causative organism

Metabolic activity	Organism											
	Salmonella Citrobacter Alcaligenes Achromobacter	*Flavobacterium Cytophaga*	*Pseudomonas putida*	*Pseudomonas aeruginosa*	*Pseudomonas fluorescens*	*Pseudomonas maltophilia*	*Serratia marsescens*	*Cloaca*	*Aeromonas*	*Proteus*
Pigment: water soluble	−	−	+	+	+	−	−	−	−	−
Pigment: water insoluble	−	+	−	−	−	(+)	+	−	−	−
Lecithinase	−	−	−	NT	+	−	+	+	+	−
Proteolysis	−	−	−	NT	+	+	(+)	(+)	++	++
H_2S production	−	−	−	NT	−	+	−	(+)	++	++
*Colourless rot** Occasional faint turbidity in the albumen										
*Yellow rot**** Yellow pigment formed in membrane at site of microbial growth										
*Fluorescent green rot*** Fluorescent green pigment throughout the albumen										
Fluorescent blue rot†* Fluorescent blue pigment throughout the albumen										
*Pink rot*** Fluorescent green albumen initially; subsequently white-pink in colour, yolk-surrounded with custard-like material										
*Green rot** Gelatinous amber-like yolk striped with olive green pigment; almond-like odour										
Red rot. White stained red throughout; yolk surrounded by custard-like material										
*Custard rot** Yolk encrusted with custard-like material and occasionally flecked with olive-green pigment										
*Black rot type 1*** Gelatinous yolk blackened throughout; grey, watery albumen										
*Black rot type 2*** Dark brown mealy yolk; dark brown albumen										

The rots were identified according to the definitions given in Florian & Trussell (1957)*, Haines (1939)**, Board & Board (1968)*** and Platt & Anderson (1939)†.

Activity: −, negative; +, weak positive; ++, strong positive; (+), although positive, considered to play no part in rotting of egg; NT, not tested.

mixtures (a CO_2/N_2 mixture ?) that will prevent growth of organisms lodged in the shell membranes.

Implicit factors. The actual physiological properties (the determinants of spoilage) that allow an organism to colonize the egg are not known in detail. All that can be stated is that these organisms are Gram-negative and have simple nutritional requirements. From the viewpoint of changes occurring during the rotting of an egg, it is possible to link the salient feature of the rot with particular physiological attributes of the causative organisms (Table 5).

Addendum

In this review no attempt has been made to interpret the antimicrobial defence in terms of its role in protecting the developing embryo. This results from the lack of systematic study as witnessed by the reports of Lutsky & Bell (1953) and Beer (1967). Such studies might well provide an answer to the question: why does a chicken turn its egg? Is it to prevent a union of the yolk and shell membranes and a concomitant 'short circuiting' of the antimicrobial defence of the albumen?

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References

Abels, J. C. (1936). The calcium binding power of egg albumen. *J. Am. chem. Soc.*, **58**: 2609-2610.

Alderton, G., Ward, W. H. & Fevold, H. L. (1946). Identification of the bacteria-inhibiting, iron-binding protein of egg white as conalbumin. *Archs Biochem.*, **11**: 9-13.

Alford, L. R., Holmes, N. E., Scott, W. J. & Vickery, J. R. (1950). Studies in the preservation of shell eggs. 1. Nature of wastage in Australian export eggs. *Aust. J. appl. Sci.*, **1**: 208-214.

Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carrs, B. & Greenberg, G. R. (1958). Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial cell walls. *J. chem. Soc.*, pp. 4344-4354.

Asbell, M. A. & Eagon, R. G. (1966). The role of multivalent cations in the organisation and structure of bacterial cell walls. *Biochem. biophys. Res. Commun.*, **22**: 664-671.

Ayres, J. C. & Taylor, B. (1956). Effect of temperature on microbial proliferation in shell eggs. *Appl. Microbiol.*, **4**: 355-359.

Baddiley, J., Buchanan, J. G. & Carrs, B. (1958). The presence of ribitol phosphate in bacterial cell walls. *Biochim. biophys. Acta*, **27**: 220.

Balls, A. K. & Swenson, T. L. (1934). The antitrypsin of egg white. *J. biol. Chem.*, **106**: 409-419.

Baumgartner, H. (1957). The effect and occurrence of avidin in raw egg white and dried egg powder. *Ernährungsforschung*, **2**: 631-634.

Bean, K. C. & MacLaury, D. W. (1959). The bacterial contamination of hatching eggs and methods for its control. *Poult. Sci.*, **38**: 693-698.

Beer, A. E. (1967). Some aspects of embryology and hatchability problems. *Poult. Rev.*, **6**: 70-75.

Berger, L. R. & Weiser, R. S. (1957). The β-glucosaminidase activity of egg-white lysozyme. *Biochim. biophys. Acta*, **26**: 517-521.

Bigland, C. H. & Papas, G. (1953). Experiment in egg penetration by *Salmonella*. *Can. J. comp. Med.*, **17**: 105-109.

Birdsell, D. C. & Cota-Robles, E. H. (1967). Production and ultra-structure of lysozyme and ethylenediaminetetra-acetate-lysozyme spheroplasts of *Escherichia coli*. *J. Bact.*, **93**: 427-437.

Board, R. G. (1962). *A study of the bacterial infection of the hen's egg*. Thesis, University of Edinburgh, Scotland.

Board, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.*, **27**: 350-364.

Board, R. G. (1965*a*). Bacterial growth on and penetration of the shell membranes of the hen's egg. *J. appl. Bact.*, **28**: 197-205.

Board, R. G. (1965*b*). The properties and classification of the predominant bacteria occurring in rotten eggs. *J. appl. Bact.*, **28**: 437-453.

Board, R. G. (1966). Review article: the course of microbial infection of the hen's egg. *J. appl. Bact.*, **29**: 319-341.

Board, R. G. & Ayres, J. C. (1965). The influence of temperature on bacterial infection of the hen's egg. *Appl. Microbiol.*, **13**: 358-364.

Board, R. G., Ayres, J. C., Kraft, A. A. & Forsythe, R. H. (1964). The microbiological contamination of egg shells and egg packing materials. *Poult. Sci.*, **43**: 584-595.

Board, P. A. & Board, R. G. (1967). A method of studying bacterial penetration of the shell of the hen's egg. *Lab. Pract.*, **16**: 471-482.

Board, P. A. & Board, R. G. (1968). A diagnostic key for identifying organisms recovered from rotten eggs. *Br. Poult. Sci.*, **9**: 111-120.

Board, P. A., Henden, L. P. & Board, R. G. (1968). The influence of iron on the course of bacterial infection of the hen's egg. *Br. Poult. Sci.* (in press).

Board, R. G. & Wilson, E. G. H. (1956). Level of microbial contamination of eggs used for hatching. *Edinb. Sch. Agric. exp. Work*, pp. 89-90.

Bowman, J. C. (1966). Storage of hatching eggs. An experiment on alternative methods of storing chicken hatching eggs. *Br. Poult. Sci.*, **7**: 219-225.

Brant, A. W. & Starr, P. B. (1962). Some physical factors related to egg spoilage. *Poult. Sci.*, **41**: 1468-1473.

Brant, A. W., Starr, P. B. & Hamann, J. A. (1966). Design of new egg washer. *Poultry Tribune*, p. 65.

Brooks, J. (1960). Mechanism of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.*, **23**: 499-509.

Brooks, J. & Taylor, D. I. (1955). Eggs and egg products. *Rep. Fd Invest. Bd*, 60. London, H.M.S.O.

Brown, W. E., Baker, R. C. & Naylor, H. B. (1965). The role of the inner shell membrane in bacterial penetration of chicken eggs. *Poult. Sci.*, **44**: 1323-1327.

Brown, W. E., Baker, R. C. & Naylor, H. B. (1966*a*). Shell treatments as affecting microbial egg spoilage. *Poult. Sci.*, **45**: 276-279.

Brown, W. E., Baker, R. C. & Naylor, H. B. (1966*b*). Egg spoilage as affected by route of exposure. *Poult. Sci.*, **45**: 279-283.

Brown, W. E., Baker, R. C. & Naylor, H. B. (1966*c*). The microbiology of cracked eggs. *Poult. Sci.*, **45**: 284-287.

Bryant, R. L. & Sharp, P. L. (1934). Effect of washing on keeping quality of hen's egg. *J. agric. Res.*, **48**: 67-89.

Buxton, A. & Gordon, R. F. (1947). The epidemiology and control of *Salmonella thompson* infection of fowls. *J. Hyg., Camb.*, **45**: 265-281.

Carson, K. T. & Eagon, R. G. (1966). Lysozyme sensitivity of the cell wall of *Pseudomonas aeruginosa*: further evidence of the role of the nonpeptidoglycon components in cell wall rigidity. *Can. J. Microbiol.*, **12**: 105-108.

Cotterill, O. J. & Gardner, F. (1957). Regarding thick white deterioration by holding shell eggs in sealed containers. *Poult. Sci.*, **36**: 196-206.

Cotterill, O. J. & Hartman, P. (1956). Effect of antibiotic on the incidence of spoilage in shell eggs. *Poult. Sci.*, **35**: 733-735.

Davis, G. T. & Beeckler, A. F. (1962). Plastic packaging of eggs. 1. Methods of packaging. *Poult. Sci.*, **41**: 391-397.

Eakin, R. E., Snell, E. E. & Williams, R. J. (1940). A constituent of raw egg white capable of inactivating biotin *in vitro*. *J. biol. Chem.*, **136**: 801-802.

Elliott, R. P. (1954). Spoilage of shell eggs by pseudomonads. *Appl. Microbiol.*, **2**: 158-163.

Elliott, R. P. & Brant, A. W. (1957). Effect of saline and egg shell membrane on bacterial growth. *Fd Res.*, **22**: 241-250.

Elliott, R. P. & Romoser, G. L. (1957). Studies on the recovery of antibiotic residues in egg albumen. *Poult. Sci.*, **36**: 365-375.

Evans, R. I., Butts, H. A. & Davidson, J. A. (1951). The vitamin B6 content of fresh and stored shell eggs. *Poult. Sci.*, **30**: 515-519.

Feeney, R. E., Ducay, E. D., Silva, R. S. & MacDonnell, L. R. (1952). Chemistry of shell egg deterioration: the egg white proteins. *Poult. Sci.*, **31**: 639-647.

Feeney, R. E. & Nagy, D. A. (1952). The antibacterial activity of the egg white protein conalbumin. *J. Bact.*, **64**: 629-643.

Ferdinandov (1944). Quoted from Zagaevsky & Lutikova (1944).

Fleming, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc.* (*B*), **93**: 306-317.

Fletcher, D. A., Orr, H. L., Snyder, E. S. & Nicholson, A. O. (1959). Effect of oiling, packaging materials and addition of CO_2 on quality of shell eggs held in storage. *Poult. Sci.*, **38**: 106-111.

Florian, M. L. E. & Trussell, P. C. (1957). Bacterial spoilage of eggs. IV. Identification of spoilage organisms. *Fd Technol., Champaign*, **11**: 56-60.

Forsythe, R. H., Ayres, J. C. & Radlo, J. L. (1953). Factors affecting the microbiological populations of shell eggs. *Fd Technol., Champaign*, **7**: 49-56.

Fraenkel-Conrat, H. & Feeney, R. E. (1950). The metal-binding activity of conalbumin. *Archs Biochem.*, **29**: 101-113.

Frank, H. & Martin, H. H. (1960). *Proceedings of the European Conference on electron microscopy, Delft.* Vol II: 1024. Amsterdam, Elsevier.

Frazier, W. C. (1967). *Food Microbiology* 2nd ed. London, McGraw-Hill.

Friedberger, E. & Hoder, F. (1932). Lysozyme und Flockungsphänomen des Hühnereiklars. *Z. Immun. Forsch. exp. Ther.*, **74**: 429-447.

Fromm, D. & Monroe, R. J. (1960). Interior physical quality and bacterial contamination of market eggs as influenced by egg shell permeability. *Fd Technol., Champaign*, **14**: 401-403.

Funk, E. M. (1943). Pasteurisation of shell eggs. *Res. Bull. Mo. agric. Exp. Stn* 364.

Funk, E. M. (1948). Experiments on cleaning soiled eggs for storage. *Res. Bull. Mo. agric. Exp. Stn* 426.

Funk, E. M., Forward, I. & Lorah, M. (1954). Maintaining quality in shell eggs by heat treatment. *Res. Bull. Mo. agric. Exp. Stn* 550.

Garibaldi, J. A. (1960). Factors in egg white which control growth of bacteria. *Fd Res.*, **25**: 337-344.

Garibaldi, J. A. & Bayne, H. G. (1960). The effect of iron on *Pseudomonas* spoilage of experimentally infected eggs. *Poult. Sci.*, **39**: 1517-1520.

Garibaldi, J. A. & Bayne, H. G. (1962*a*). Iron and bacterial spoilage of shell eggs. *J. Fd Sci.*, **27**: 57-59.

Garibaldi, J. A. & Bayne, H. G. (1962*b*). The effect of iron on *Pseudomonas* spoilage of farm washed eggs. *Poult. Sci.*, **41**: 850-853.

Garibaldi, J. A. & Stokes, J. L. (1958). Protective role of shell membranes in bacterial spoilage of eggs. *Fd Res.*, **23**: 283-290.

Ghosh, B. K. & Murray, R. G. E. (1967). Fine structure of *Listeria monocytogenes* in relation to protoplast formation. *J. Bact.*, **93**: 411-426.

Gillespie, J. H. & Scott, W. J. (1950). Studies in the preservation of shell eggs. IV. Experiments on the mode of infection by bacteria. *Aust. J. appl. Sci.*, **1**: 514-530.

Gillespie, J. H., Scott, W. J. & Vickery, J. R. (1950*a*). Studies in the preservation of shell eggs. II. The incidence of bacterial rotting in unwashed eggs and in eggs washed by hand. *Aust. J. appl. Sci.*, **1**: 215-223.

Gillespie, J. H., Scott, W. J. & Vickery, J. R. (1950*b*). Studies in the preservation of shell eggs. III. The storage of machine washed eggs. *Aust. J. appl. Sci.*, **1**: 313-329.

Gillespie, J. H., Salton, M. R. J. & Scott, W. J. (1950). Studies in the preservation of shell eggs. V. The use of chemical disinfectants in cleaning machines. *Aust. J. appl. Sci.*, **1**: 531-538.

Gordon, R. F. & Tucker, J. F. (1954). Behaviour of *Pseudomonas* sp. and the natural occurrence of the organism in the fowl and its environment. *Contribution 109 of the 10th Wld's Poult. Sci. Congr. Edinburgh*: Department of Agriculture, Scotland.

Gordon, R. F. & Tucker, J. F. (1965). The epizootology of *Salmonella menston* infection of fowls and the effect of feeding poultry food infected with *Salmonella*. *Br. Poult. Sci.*, **6**: 251-264.

Goresline, H. E., Moser, R. E. & Hayes, K. M. (1950). A pilot scale study of shell egg thermostabilization. *Fd Technol., Champaign*, **4**: 426-430.

Graves, R. C. & MacLaury, D. W. (1962). The effects of temperature, vapour pressure and absolute humidity on bacterial contamination of shell eggs. *Poult. Sci.*, **41**: 1219-1225.

Gray, G. W. & Wilkinson, S. G. (1965*a*). The action of ethylenediamine-tetra-acetic acid on *Pseudomonas aeruginosa*. *J. appl. Bact.*, **28**: 153-164.

Gray, G. W. & Wilkinson, S. G. (1965*b*). The action of ethylenediaminetetra-acetic acid on the cell walls of some Gram-negative bacteria. *J. gen. Microbiol.*, **39**: 385-389.

Hadley, P. B. & Caldwell, D. W. (1916). The bacterial infection of fresh eggs. *Bull. Rhode Isl. agric. Exp. Stn* 164.

Haines, R. B. (1938). Observations on the bacterial flora of the hen's egg, with a description of a new species of *Proteus* and *Pseudomonas* causing rots in eggs. *J. Hyg., Camb.*, **38**: 338-355.

Haines, R. B. (1939). Microbiology in the preservation of the hen's egg. *Rep. Fd Invest. Bd*, 47. London, H.M.S.O.

Haines, R. B. & Moran, T. (1940). Porosity of, and bacterial invasion through, the shell of the hen's egg. *J. Hyg., Camb.*, **40**: 453-461.

Hale, H. P. (1950). The production of 'amber whites' in shell eggs by freezing and thawing. *J. Sci. Fd Agric.*, **1**: 46-48.

Harry, E. G. (1957). The effect on embryonic and chick mortality of yolk contamination with bacteria from the hen. *Vet. Rec.*, **69**: 1433-1441.

Harry, E. G. (1963*a*). Some observations on the bacterial content of the ovary and oviduct of the fowl. *Br. Poult. Sci.*, **4**: 63-90.

Harry, E. G. (1963*b*). The relationship between egg spoilage and the environment of the egg when laid. *Br. Poult. Sci.*, **4**: 91-100.

Hartung, T. E. & Stadelman, W. T. (1962). Influence of metallic ions on the penetration of the egg shell membranes by *Pseudomonas fluorescens*. *Poult. Sci.*, **41**: 1590-1596.

Hartung, T. E. & Stadelman, W. T. (1963). *Pseudomonas fluorescens* penetration of egg shell membranes as influenced by shell porosity, age of egg and degree of bacterial challenge. *Poult. Sci.*, **42**: 147-150.

Healey, D. J. & Peter, A. M. (1925). The hydrogen ion concentrations and basicity of egg yolk and egg white. *Am. J. Physiol.*, **74**: 363-368.

Heater, C. D. & Zant, W. C. van der (1957). Effects of plating medium on the survival of heat treated cells of *Pseudomonas fluorescens*. *Fd Res.*, **22**: 164-169.

Kellenberger, E. & Ryter, A. (1958). Cell wall and cytoplasmic membrane of *Escherichia coli*. *J. biophys. biochem. Cytol.*, **4**: 323-326.

Knowles, N. R. (1956). The prevention of microbial spoilage in whole shell eggs by heat treatment methods. *J. appl. Bact.*, **19**: 293-300.

Knowles, N. R. (1957*a*). Observations on the keeping quality of dry-cleaned eggs. *Res. exp. Rec. Minist. Agric. Nth. Ire.*, **6**: 100-107.

Knowles, N. R. (1957*b*). The cold storage of dry-cleaned eggs. *Res. exp. Rec. Minist. Agric. Nth. Ire.*, **6**: 108-117.

Kohn, A. (1960). Lysis of frozen and thawed cells of *Escherichia coli* by lysozyme, and their conversion into spheroplasts. *J. Bact.*, **79**: 697-706.

Korotkova, G. P. (1957). On the protective function of the albuminous membrane in the hen's egg. *Zh. obshch. Biol.*, **18**: 275-287.

Kraft, A. A., Elliott, L. E. & Brant, A. W. (1958). The shell membrane as a barrier to bacterial penetration of eggs. *Poult. Sci.*, **37**: 238-240.

Kraft, A. A., McNally, E. G. & Brant, A. W. (1958). Shell quality and bacterial infection of shell eggs. *Poult. Sci.*, **37**: 638-643.

Lancaster, J. E. & Crabb, W. E. (1953). Studies on disinfection of eggs and incubators. 1. The survival of *Salmonella pullorum*, *thompson* and *typhi-murium* on the surface of the hen's egg. *Br. vet. J.*, **109**: 139-148.

Laschtschenko, P. (1909). Über die keimtötende und entwicklungshemmende Wirkung Huhnereiweiss. *Z. Hyg. InfektKrankh.*, **64**: 419-427.

Lifshitz, A., Baker, R. C. & Naylor, H. B. (1964). The relative importance of chicken egg exterior structures in resisting bacterial penetration. *J. Fd Sci.*, **29**: 94-99.

Lifshitz, A., Baker, R. C. & Naylor, H. B. (1965). The exterior structures of the egg as nutrients for bacteria. *J. Fd Sci.*, **30**: 516-519.

Lineweaver, H. & Murray, C. W. (1947). Identification of the trypsin inhibitor of egg white with ovomucoid. *J. biol. Chem.*, **171**: 565-581.

Longsworth, L. G., Cannan, R. K. & MacInnes, D. A. (1940). The electrophoretic analysis of egg white. *J. Am. chem. Soc.*, **62**: 2580-2590.

Lorenz, F. W., Starr, P. B., Starr, M. P. & Ogasawara, F. X. (1952). The development of *Pseudomonas* spoilage in eggs. 1. Penetration through the shell. *Fd Res.*, **17**: 351-360.

Lutsky, I. I. & Bell, A. E. (1953). Antibacterial action of egg albumen and embryonic immunity. *Poult. Sci.*, **32**: 285-293.

Magwood, S. E. (1964). Studies in hatchery sanitation. 3. The effect of air-borne bacterial populations on contamination of egg and embryo surfaces. *Poult. Sci.*, **43**: 1567-1572.

Marshall, W. & Cruikshank, D. B. (1938). The function of the cuticle in relation to the porosity of eggs. *J. agric. Sci., Camb.*, **28**: 24-42.

Martin, H. H. (1963). Bacterial protoplasts—a review. *J. theor. Biol.*, **5**: 1-34.

Masushima, K. (1958). An undescribed trypsin inhibitor in egg white. *Science, N.Y.*, **127**: 1178-1179.

May, H. G. (1924). The examination of eggs from infected and immunized hens, with germicidal tests on albumen and blood serum. *Bull. Rhode Isl. agric. Exp. Stn* 197.

McNally, E. H. (1953). Some factors related to the incidence of bacterial infection of shell eggs. *Poult. Sci.*, **32**: 915.

Miles, A. A. & Halnan, E. T. (1937). A new species of micro-organism (*Proteus melanovogenes*) causing black rot in eggs. *J. Hyg., Camb.*, **37**: 79-97.

Miller, W. A. & Crawford, L. B. (1953). Some factors influencing bacterial population of eggs. *Poult. Sci.*, **32**: 303-309.

Murphy, T. W. & Sutton, W. S. (1947). The pasteurisation of egg shells to prevent storage rot and maintain quality. *Agric. Gaz. N.S.W. Misc. Publ.* 3317.

Nermut, M. V. & Murray, R. G. E. (1967). Ultrastructure of the cell wall of *Bacillus polymyxa*. *J. Bact.*, **93**: 1949-1965.

Nelson, F. E. (1943). Factors which influence the growth of heat-treated bacteria. 1. Comparison of four agar media. *J. Bact.*, **45**: 395-403.

Orel, V. (1959). The *Pseudomonas* spoilage of eggs laid by individual hens. *Poult. Sci.*, **38**: 8-12.

Pathak, R. C., Singh, C. M. & Tangri, R. P. (1960). Chick mortality and the contamination of yolks by members of the Enterobacteriaceae. *Br. vet. J.*, **116**: 81-84.

Paton, A. M. & Ayres, J. C. (1964). Use of a fluorescent brightener for tracing the passage of *Salmonella* in eggs. *Nature, Lond.*, **204**: 803-804.

Perkins, H. R. & Rogers, H. J. (1959). The products of the partial hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. *Biochem. J.*, **72**: 647-654.

Platt, A. E. & Anderson, C. F. (1939). Green rot in eggs. *J. Dept. Agric. S. Aust.*, **42**: 1040-1041.

Proudfoot, F. G. (1964). The effects of plastic packaging and other treatments on hatching eggs. *Can. J. anim. Sci.*, **44**: 87-95.

Proudfoot, F. G. (1965). The effect of film permeability and concentration of nitrogen, oxygen and helium gases on hatching eggs stored in polyethylene and cryovac bags. *Poult. Sci.*, **44**: 636-644.

Proudfoot, F. G. (1966*a*). Hatchability of stored chicken eggs as affected by daily turning during storage and pre-warming and vacuuming eggs enclosed in plastic with nitrogen. *Can. J. anim. Sci.*, **46**: 47-50.

Proudfoot, F. G. (1966*b*). The use of sealed cryovac and polyethylene case liners with and without nitrogen gas for the preservation of hatching eggs. *Poult. Sci.*, **45**: 105-108.

Reid, W. M., Macy, T. A., Boyd, F. M., Kleckner, A. L. & Schmittle, S. C. (1961). Embryo and baby chick mortality and morbidity induced by a strain of *Escherichia coli*. *Poult. Sci.*, **40**: 1497-1502.

Repaske, R. (1956). Lysis of Gram-negative bacteria by lysozyme. *Biochim. biophys. Acta*, **22**: 189-191.

Repaske, R. (1958). Lysis of Gram-negative organisms and the role of versene. *Biochim. biophys. Acta*, **30**: 225-232.

Rettger, L. F. (1913). The bacteriology of the hen's egg, with special reference to its freedom from microbic invasion. *Bull. Storrs. agric. Exp. Stn* 75.

Rhodes, M. B., Bennett, N. & Feeney, R. E. (1959). The flavoprotein-apoprotein system of egg white. *J. biol. Chem.*, **234**: 2054-2060.

Rhodes, M. B., Bennett, N. & Feeney, R. E. (1960). The trypsin and chymotrypsin inhibitors from avian egg whites. *J. biol. Chem.*, **235**: 1686-1693.

Richard, O. & Mohler, A. (1950). Die Mikrobiologie der 'Heu-eier'. *Mitt. Lebensm. Hyg., Bern.*, **41**: 168-180.

Rizk, S. S., Ayres, J. C. & Kraft, A. A. (1966*a*). Disinfection of eggs artificially inoculated with *Salmonella*. 1. Application of several disinfectants. *Poult. Sci.*, **45**: 764-769.

Rizk, S. S., Ayres, J. C. & Kraft, A. A. (1966*b*). Effect of holding condition on the development of *Salmonellae* in artificially inoculated hen's eggs. *Poult. Sci.*, **45**: 825-829.

Rogers, H. J. (1965). The outer layers of bacteria: the biosynthesis of structure. In *Function and structure in micro-organisms*, Symp. Soc. gen. microbiol. no. 15. pp. 186-219. Edit. Pollock, M. R. and Richmond, M. H. Cambridge, University Press.

Romanoff, A. L. & Romanoff, A. J. (1944). A study of preservation of eggs by flash heat treatment. *Fd Res.*, **9**: 358-366.

Romanoff, A. L. & Romanoff, A. J. (1949). *The avian egg*. New York, Wiley and Sons.

Rosser, F. T. (1942). Preservation of eggs. II. Surface contamination on egg-shell in relation to spoilage. *Can. J. Res*, **20D**: 291-296.

Salton, M. R. J. (1957). The properties of lysozyme and its action on micro-organisms. *Bact. Rev.*, **21**: 82-99.

Salton, M. R. J. (1964). *The bacterial cell wall*. Amsterdam, Elsevier.

Salton, M. R. J. & Ghuysen, J. M. (1960). Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. III. The structure of di- and tetra-saccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme. *Biochim. biophys. Acta*, **45**: 355-363.

Salton, M. R. J., Scott, W. J. & Vickery, J. R. (1951). Studies in the preservation of shell eggs. IV. The effect of pasteurisation on bacterial rotting. *Aust. J. appl. Sci.*, **2**: 205-222.

Sauter, E. A. (1966). The effect of lye on the incidence of fluorescent spoilage in washed eggs. *Poult. Sci.*, **45**: 131-135.

Sauter, E. A., Petersen, C. F. & Lampman, C. E. (1962). The effectiveness of various sanitizing agents in the reduction of green rot spoilage in washed eggs. *Poult. Sci.*, **41**: 468-473.

Schade, A. L. (1958). Siderophilin and conalbumin as physiologically active iron-chelating proteins. *Abstr. Proc. Int. Congr. Biochem., 4th Cong. Vienna*, p. 127, 10-42.

Schade, A. L. (1961). The microbiological activity of siderophilin. *Protides of the biological fluids*, pp. 261-263. *Proc. 8th Coll., Bruges*. Amsterdam, Elsevier.

Schade, A. L. (1963). Significance of serum iron for the growth, biological characteristics, and metabolism of *Staphylococcus aureus*. *Biochem. Z.*, **338**: 140-148.

Schade, A. L. & Caroline, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science, N.Y.*, **100**: 14-15.

Schaible, F. J., Bandemer, S. L. & Davidson, J. A. (1946). Composition of fresh and storage eggs from hens fed cottonseed and non cottonseed rations. I. General observations. *Poult. Sci.*, **25**: 440-445.

Schmidt, F. J. & Stadelman, W. J. (1957). Effects of antibiotics and heat treatment of shell eggs on quality after storage. *Poult. Sci.*, **36**: 1023-1026.

Scott, W. J. & Vickery, J. R. (1954). Studies on the preservation of eggs. VII. The effect of pasteurisation on the maintenance of physical quality. *Aust. J. appl. Sci.*, **5**: 89-102.

Sharp, P. F. & Stewart, G. F. (1936). Effect of relative humidity on the growth of moulds on eggs in storage. *Mem. Cornell Univ. agric. Exp. Stn* **91**.

Sharp, P. F. & Whitaker, R. (1927). The relation of the hydrogen ion concentration of egg white to its germicidal action. *J. Bact.*, **14**: 17-46.

Simkiss, K. (1961). Calcium metabolism and avian reproduction. *Biol. Rev.*, **36**: 321-367.

Simons, P. C. M. & Wiertz, G. (1966). The ultra-structure of the surface of the cuticle of the hen's egg in relation to egg cleaning. *Poult. Sci.*, **45**: 1153-1162.

Stokes, J. L. & Osborne, W. W. (1956). Effect of the egg shell membrane on bacteria. *Fd Res.*, **21**: 264-269.

Stokes, J. L., Osborne, W. W. & Bayne, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Fd Res.*, **21**: 510-518.

Stuart, L. S. & McNally, E. H. (1943). Bacteriological studies on the egg shell. *U.S. Egg Poult. Mag.*, **49**: 28-31, 45-47.

Theodore, T. S. & Schade, A. L. (1965*a*). Growth of *Staphylococcus aureus* in media of restricted and unrestricted inorganic iron availability. *J. gen. Microbiol.*, **39**: 75-83.

Theodore, T. S. & Schade, A. L. (1965*b*). Carbohydrate metabolism of iron-rich and iron-poor *Staphylococcus aureus*. *J. gen. Microbiol.*, **40**: 385-396.
Thompson, R. (1940). Lysozyme and its relation to anti-bacterial properties of various tissues and secretions. *Archs Path.*, **30**: 1096-1134.
Thompson, R. (1941). Lysozyme and the anti-bacterial properties of tears. *Archs Ophthal., N.Y.*, **25**: 491-509.
Trussell, P. C., Fulton, C. O. & Cameron, C. I. (1955). Bacterial spoilage of shell eggs. II. Incidence of spoilage in eggs from ninety-four farms. *Fd Technol., Champaign*, **9**: 130-134.
Trussell, P. C., Triggs, R. E. & Greer, B. A. (1955). Bacterial spoilage of egg shells. III. Farm practices promoting spoilage. *Fd Technol., Champaign*, **9**: 134-137.
Tyler, C. (1945). The porosity of egg shells and the influence of different levels of dietary calcium upon porosity. *J. agric. Sci., Camb.*, **35**: 168-176.
Tyler, C. (1953). Studies on egg shells. II. A method for marking and counting pores. *J. Sci. Fd Agric.*, **4**: 266-272.
Tyler, C. (1956). Studies on egg shells. VII. Some aspects of structure as shown by plastic models. *J. Sci. Fd Agric.*, **7**: 483-493.
Vos, J. C. (1964). Lysozyme lysis of Gram-negative bacteria without production of spheroplasts. *J. gen. Microbiol.*, **35**: 313-317.
Walden, C. C., Allen, I. V. F. & Trussell, P. C. (1956). The role of the egg shell and shell membranes in restraining the entry of microorganisms. *Poult. Sci.*, **35**: 1190-1196.
Warren, D. C., Roff, H. A. & Long, E. (1965). Hatchability of eggs stored in plastic-lined egg cases. *Poult. Sci.*, **44**: 1278-1280.
Weibull, C. (1953*a*). The isolation of protoplasts from *Bacillus megatherium* by controlled treatment with lysozyme. *J. Bact.*, **66**: 688-695.
Weibull, C. (1953*b*). Characterization of the protoplasmic constituents of *Bacillus megatherium*. *J. Bact.*, **66**: 696-702.
Weidel, W. & Pelzer, H. (1964). Bag shaped macromolecules—a new outlook on bacterial cell walls. *Adv. Enzymol.*, **26**: 193-232.
Weinbaum, G. & Markman, R. (1966). A rapid technique for distinguishing enzymatically active proteins in cell 'envelopes' of *Escherichia coli*. *Biochim. biophys. Acta*, **124**: 207-209.
Weinbaum, G., Rich, R. & Fischman, D. A. (1967). Enzyme-induced formation of spheres from cells and envelopes of *Escherichia coli*. *J. Bact.*, **93**: 1693-1698.
Williams, J. (1962). Serum proteins and livetins of hen's egg yolk. *Biochem. J.*, **83**: 355-364.
Wilson, J. E. (1945). Infected egg shells are a means of spread of salmonellosis in chicks and ducklings. *Vet. Rec.*, **57**: 411-413.
Winter, A. R., Burhart, B. & Wettling, C. (1952). Cleaning eggs for market. *Bull. Ohio agric. Exp. Stn* 710.
Winter, A. R., Burkart, B., Clements, P. & MacDonald, L. (1955). Cleaning eggs with detergents and detergent-sanitizers. *Bull. Ohio agric. Exp. Stn* 762.
Wolin, M. J. (1966). Lysis of *Vibrio succinogenes* by ethylene diaminetetra-acetic acid or lysozyme. *J. Bact.*, **91**: 1781-1786.
Wolk, J., McNally, E. H. & Spicknall, N. H. (1950). The effect of temperature on bacterial infection of shell eggs. *Fd Technol., Champaign*, **4**: 316-318.
Woolley, D. W. & Longsworth, L. G. (1942). Isolation of an anti-biotin factor from egg white. *J. biol. Chem.*, **142**: 285-290.
Work, E. (1957). Biochemistry of the bacterial cell wall. *Nature, Lond.*, **179**: 841-847.
Zagaevsky, J. S. & Lutikova, P. O. (1944). Sanitary measures in egg breaking plant. *U.S. Egg Poult. Mag.*, **50**: 17-20, 43-46, 75-77, 89-90, 121-123.
Zinder, N. D. & Arndt, W. F. (1956). Production of protoplasts of *Escherichia coli* by lysozyme treatment. *Proc. natn. Acad. Sci. U.S.A.*, **42**: 586-590.

Romanoff, A. L. & Romanoff, A. J. (1944). A study of preservation of eggs by flash heat treatment. *Fd Res.*, **9**: 358-366.

Romanoff, A. L. & Romanoff, A. J. (1949). *The avian egg*. New York, Wiley and Sons.

Rosser, F. T. (1942). Preservation of eggs. II. Surface contamination on egg-shell in relation to spoilage. *Can. J. Res*, **20D**: 291-296.

Salton, M. R. J. (1957). The properties of lysozyme and its action on micro-organisms. *Bact. Rev.*, **21**: 82-99.

Salton, M. R. J. (1964). *The bacterial cell wall*. Amsterdam, Elsevier.

Salton, M. R. J. & Ghuysen, J. M. (1960). Acetylhexosamine compounds enzymically released from *Micrococcus lysodeikticus* cell walls. III. The structure of di- and tetra-saccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme. *Biochim. biophys. Acta*, **45**: 355-363.

Salton, M. R. J., Scott, W. J. & Vickery, J. R. (1951). Studies in the preservation of shell eggs. IV. The effect of pasteurisation on bacterial rotting. *Aust. J. appl. Sci.*, **2**: 205-222.

Sauter, E. A. (1966). The effect of lye on the incidence of fluorescent spoilage in washed eggs. *Poult. Sci.*, **45**: 131-135.

Sauter, E. A., Petersen, C. F. & Lampman, C. E. (1962). The effectiveness of various sanitizing agents in the reduction of green rot spoilage in washed eggs. *Poult. Sci.*, **41**: 468-473.

Schade, A. L. (1958). Siderophilin and conalbumin as physiologically active iron-chelating proteins. *Abstr. Proc. Int. Congr. Biochem., 4th Cong. Vienna*, p. 127, 10-42.

Schade, A. L. (1961). The microbiological activity of siderophilin. *Protides of the biological fluids*, pp. 261-263. *Proc. 8th Coll., Bruges*. Amsterdam, Elsevier.

Schade, A. L. (1963). Significance of serum iron for the growth, biological characteristics, and metabolism of *Staphylococcus aureus*. *Biochem. Z.*, **338**: 140-148.

Schade, A. L. & Caroline, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science, N.Y.*, **100**: 14-15.

Schaible, F. J., Bandemer, S. L. & Davidson, J. A. (1946). Composition of fresh and storage eggs from hens fed cottonseed and non cottonseed rations. 1. General observations. *Poult. Sci.*, **25**: 440-445.

Schmidt, F. J. & Stadelman, W. J. (1957). Effects of antibiotics and heat treatment of shell eggs on quality after storage. *Poult. Sci.*, **36**: 1023-1026.

Scott, W. J. & Vickery, J. R. (1954). Studies on the preservation of eggs. VII. The effect of pasteurisation on the maintenance of physical quality. *Aust. J. appl. Sci.*, **5**: 89-102.

Sharp, P. F. & Stewart, G. F. (1936). Effect of relative humidity on the growth of moulds on eggs in storage. *Mem. Cornell Univ. agric. Exp. Stn* 91.

Sharp, P. F. & Whitaker, R. (1927). The relation of the hydrogen ion concentration of egg white to its germicidal action. *J. Bact.*, **14**: 17-46.

Simkiss, K. (1961). Calcium metabolism and avian reproduction. *Biol. Rev.*, **36**: 321-367.

Simons, P. C. M. & Wiertz, G. (1966). The ultra-structure of the surface of the cuticle of the hen's egg in relation to egg cleaning. *Poult. Sci.*, **45**: 1153-1162.

Stokes, J. L. & Osborne, W. W. (1956). Effect of the egg shell membrane on bacteria. *Fd Res.*, **21**: 264-269.

Stokes, J. L., Osborne, W. W. & Bayne, H. G. (1956). Penetration and growth of *Salmonella* in shell eggs. *Fd Res.*, **21**: 510-518.

Stuart, L. S. & McNally, E. H. (1943). Bacteriological studies on the egg shell. *U.S. Egg Poult. Mag.*, **49**: 28-31, 45-47.

Theodore, T. S. & Schade, A. L. (1965*a*). Growth of *Staphylococcus aureus* in media of restricted and unrestricted inorganic iron availability. *J. gen. Microbiol.*, **39**: 75-83.

CHAPTER 2

Plasma and Egg White Proteins

R. G. Board and D. J. Hornsey

I. General Introduction

If a class of vertebrates is not included regularly in the studies leading to the elucidation of a fundamental aspect of physiology, then there is a tendency for the occasional investigation to attempt to establish links between the neglected class and the main body of information. This tendency is obvious with the plasma proteins of birds and is reflected in this chapter where organization and interpretation have been influenced by analogy to the plasma proteins of mammals.

An entirely different situation occurs with egg white proteins mainly because an elementary skill—the ability to break the shell and harvest the albumen without contamination with yolk material—is the only one to be mastered before a solution of proteins with no cellular organization, negligible enzyme activity, and little contamination with nonprotein material is obtained. Thus it is little wonder that eggs were a popular source of proteins at every phase in the evolution of methods for the isolation and characterization of proteins. An additional bonus, of course, is to have a solution of proteins, many of which have some easily demonstrated property or function. The egg white proteins exhibit these features (Table I). There are no grounds for complaint

TABLE I

SOME BIOLOGICAL AND PHYSICAL PROPERTIES OF THE MAIN PROTEINS OF THE ALBUMEN OF THE HEN'S EGG

Protein	Amount (%) in albumen	MW	p*I*	Biological property	Methods of assay
1. Ovalbumin	54	46,000	4.5	—	—
2. Ovotransferrin	12	76,600	6.05	Binding [2 atoms(mole^{-1})] of Fe^{3+}, Cu^{2+}, Mn^{2+} Co^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+}	Spectral studies (Tan and Woodworth, 1968; Phelps and Antonini, 1975); growth studies with microbes (Theodore and Schade, 1965a,b)
				Binding of bicarbonate	
3. Ovomucoid	12	28,000	4.1	Inhibition of proteases	Standard biochemical techniques
				Anaphylactoid response in rats	Injection of albino rats
4. Lysozyme	3.4	14,300	10.7	Hydrolysis of $\beta(1-4)$ glycosidic bond in peptidoglycans	Spectral studies with particulate substrate (cell walls of *Micrococcus lysodeikticus*)
				Scaffolding of yolk/embryo through electrostatic interaction with ovomucin	Rheological methods
5. Ovomucin	3.5	*See 4* (Above)	4.5–5.0	Scaffolding of yolk/embryo through interaction with lysozyme	Rheological methods
				Virus antihemagglutination	Serological methods
6. Ovoinhibitor	1.5	44/49,000	5.1	Inhibition of proteases	Standard biochemical techniques
7. Ovomacroglobulin	0.5	900,000	4.5	—	—
8. Ovoglycoprotein	1.0	24,400	3.9	—	—
9. Ovoflavoprotein	0.8	32,000	4.0	Binding of riboflavin	Microbiological assay
10. Avidin	0.05	68,300	10	Binding of biotin	Microbiological assay

about the information available on the properties and characteristics of the egg white proteins. The same cannot be said about their function in the egg. The Editor's invitation to speculate has not been neglected in the discussion of novel roles of the egg white proteins. If this provokes studies of biological function, then the speculation will not have been in vain. The choice of references is another feature of the second part of the chapter which needs comment. Their selection was dictated by two main considerations: (1) to provide, as far as possible, references to recent papers so that adequate guidance to the literature was provided, and (2) to emphasize that the avian egg is more often than not the source of materials rather than a subject of study in its own right.

II. Plasma Proteins

A. Prealbumin

Marshall and Deutsch (1950), Heim and Schechtman (1954), and Vanstone *et al.* (1955) all reported the presence of prealbumin electrophoretic bands in chick embryo serums up to day 1 posthatch, with maximum concentrations of 0.14 gm% appearing at day 18 of incubation. Christov and Rashev (1961) confirmed their presence by day 6 but their disappearance by day 15 of incubation. Electrophoretic analysis is open to these aberrational differences, for the stated presence or absence of protein peaks is quite often dependent upon the buffer and pH employed (Table I) (Moore *et al.*, 1945; Marshall and Deutsch, 1950).

The prealbumin bands are rich in phospholipids (Marshall and Deutsch, 1950) and may play some part in the transport of yolk lipid reserves necessary for the developing embryo (Nobel and Moore, 1966). During egg production, 18-month-old laying hens show an electrophoretically fast moving component (Brandt *et al.*, 1952) which was correlated to egg formation when there is a mobilization of reserves. Prealbumin fractions have been confirmed in laying hens (Lush, 1963) and shown to be at maximum concentration when the shell is produced in the uterus (Kristjansson *et al.*, 1963). Several prealbumin bands have been detected in adult peafowl serum (Kimura *et al.*, 1970), pigeon serum (Baxendale *et al.*, 1971), and other species (Table II). Gel filtration chromatography indicates that these are probably low molecular weight (10,000 daltons) proteins (Baxendale *et al.*, 1971).

Although synthesis of the prealbumins is genetically determined by one autosomal locus with two alleles designated Pa^A and Pa^B (Stratil,

TABLE II

ELECTROPHORETIC MOBILITY DATA FOR AVIAN AND HUMAN SERUM COMPONENTS IN THREE BUFFERS[a]

Animal	Measurement[a]	Prealbumin	Albumin	Globulin α_1	Globulin α_2	Globulin β_1	Globulin β_2	Globulin γ	Fibrinogen
Veronal–citrate buffer, pH 8.6., $\mu = 0.1$									
Man	m		6.6	5.4	4.3	3.1		1.3	2.3
	%		59.6	6.7	8.8	11.0		9.1	4.8
Chicken	m	8.1	7.3	6.1	4.6			2.9	
	%	0.5	38.2	15.8	7.7			37.5	
Duck	m	7.6	6.7	5.8	4.9			2.5	3.7
	%	2.6	47.8	21.9	6.1			6.0	15.5
Pheasant	m	6.1	5.2	4.2	3.6			1.7	2.9
	%	0.4	58.2	14.0	6.5			4.3	16.3
Pigeon	m	7.8	6.4	5.2	4.5			1.7	3.3
	%	3.1	64.1	7.2	4.5			7.7	17.4
Turkey	m	6.7	5.9	5.0	4.1			1.7	2.9
	%	1.0	51.5	13.4	4.3			8.1	21.6
Veronal buffer, pH 8.6, $\mu = 0.1$									
Man	m		5.9	5.1	4.1	2.8		1.0	
	%		63.0	5.0	7.0	13.0		12.0	
Chicken	m		5.7	4.5		3.5		1.9	
	%		58.0	16.0		9.0		17.0	
Pigeon	m		5.8	4.4	2.8		2.4	1.7	
	%		58.0	10.0	5.0		18.0	9.0	
Phosphate buffer, pH 7.4, $\mu = 0.2$									
Man	m		5.1		3.5	2.5		0.7	
	%		65.0		9.0	16.0		11.0	
Chicken (male)	m		5.2	4.2	3.2	2.3		1.5	
	%		44.0	15.0	10.0	14.0		14.0	
Chicken (female)	m	5.8	4.9	4.0	3.1	1.8		1.1	
	%	5.0	29.0	6.0	8.0	35.0		21.0	
Pigeon	m		4.1		2.2	1.6		0.3	
	%		57.0		10.0	16.0		8.0	

1970), Asofsky *et al.* (1962) suggested that they may not be synthesized in the embryo. It has generally been assumed (Butler, 1971) that prealbumins are transferred to the embryo via the yolk. Wise *et al.* (1964), noting the similarities of alkaline phosphatase reactions on proteins from sera and egg proteins, considered prealbumins as ovalbumins and thus components of egg white and not the yolk. Because prealbumins are present from at least day 11 of incubation and before the developing chick has swallowed egg white proteins, Wise *et al.* (1964) suggested that prealbumin synthesis does occur in the embryo. Such an observation would tend to indicate a positive function of these proteins in the embryo and not a "contaminant" from the egg.

B. Albumin

Although intensive biochemical and chemical investigations have been applied to the study of albumin, the compound still has no well-established biological function. In birds, its amino acid composition (Schjeide, 1963) and its molecular weight of 65,000 daltons show it to be similar to mammalian albumin. It probably consists of a single polypeptide chain. In common with mammals, the albumin from bird species has aspartic acid as the N-terminal amino acid (Peters *et al.*, 1958; Brown, 1975). By definition (Hughes, 1954) the molecule contains no carbohydrate, any being detected on crystallization usually being assigned to small amounts of α-globulins present as contaminants. The albumin molecule is stabilized by disulfide cross-linking (40 sulfurs per 65,000 gm) and, providing these links are unaltered, there may be a considerable amount of reversible configurational variation in the molecule. The C-terminal amino acid residues of three avian species (Table III) may be compared with some mammalian data (Peters *et al.*, 1958). Alanine is the C-terminal amino acid in two

TABLE III
C- AND N-TERMINAL AMINO ACIDS OF ALBUMINS IN THREE AVIAN AND TWO MAMMALIAN SPECIES

Species	Order	N-Terminal	C-Terminal					
			6	5	4	3	2	1
Species								
Duck	Anseriformes	Asp–	(Thr,	Ser,	Leu,	Val,	Gly)-	Ala
Chicken	Galliformes	Asp–	(Ser,	Thr,	Val,	Leu,	Gly)-	Ala
Turkey	Galliformes	Asp–		(Ala,	Thr,	Leu,	Gly)-	Val
Mammalia								
Cow	Artiodactylia	Asp–	(Ala,	Ser,	Val,	Thr,	Leu)-	Ala
Man	Primata	Asp–			(Gly,	Val,	Ala)-	Leu

different orders of birds (Anseriforme and Galliforme) but in the turkey (a Galliforme) is replaced by valine.

Two or more serum albumins have been reported in fowl (McIndoe, 1962), in turkey (Quinteros *et al.*, 1964), and in ducklings under immobilization stress (Paulov, 1972). Such polymorphisms of the molecule are not reflected in any differences in their physicochemical properties (Fried and Chum, 1971).

Albumin is a highly charged molecule with a great affinity for ions, particularly anions. The binding of a large number of organic anions is probably explained by the reversible configurations within the molecule (Karush, 1950) and one of the functions of the molecule may be the transport of both desirable and nondesirable ions through the body (Foster, 1960). Fatty acid transport is considered to be one such important role (Fredrickson and Gordon, 1958). Although there are considerable structural similarities between mammalian and avian albumin, the binding patterns and sites may vary with different species. This is so for uric acid (Simkin, 1972) and for thyroxine (T_4) and triiodothyronine (T_3) (Tata and Shellaburger, 1959).

Up to the sixth day of incubation, embryo serum albumin is supplied from the yolk reservoir (Nace, 1953; Zaccheo and Grossi, 1967) and is manufactured by the yolk sac (Gitlin and Kitzes, 1967). This yolk-derived albumin may have a proliferative effect on liver cells (Konyshev, 1968) and, by day 6 of incubation, albumin is supplied by the liver (Nace, 1953), reaching a serum concentration of 0.21% (Christov and Rashev, 1961). By day 21 of incubation, concentrations are 0.62% and on day 3 of posthatch, 0.87% (Christov and Rashev, 1961). Thereafter, albumin levels remain constant (Brandt *et al.*, 1952).

C. Globulins

The conventional mammalian nomenclature of α- and β-globulin arises from electrophoretic studies. Such proteins are heterogeneous, and in the following discussion an attempt will be made to place them in a more homogeneous context.

1. Lipoproteins

Lipid transport systems of birds, in particular their lipoproteins, are in general less complicated than those in mammals (Hillyard *et al.*, 1972). In mammals exogenous fat (triglycerides) is transported in chylomicrons via the lymph. In chickens, chylomicrons are not formed and exogenous fat is transported as very low-density lipoproteins (VLDL) via the portal vein (Noyan *et al.*, 1964). Mammalian chylomicrons are the least dense of the plasma lipoproteins and are particulate (0.075–1 μm diameter) and by definition are found in chyle which is

formed in the lymphatic system draining the intestine (Mayes, 1973). The chylomicrons reported in the rooster (Schjeide and Urist, 1956) are a convenience term by these authors referring to lipoproteins of density 1.003 gm cm^{-3} and may not be related to mammalian chylomicrons.

Three weeks prior to the onset of laying, serum VLDL is increased from trace levels to 2 gm% as a result of increased estrogen levels (Hillyard *et al.*, 1956) stimulating liver synthesis. This accumulation of serum VLDL serves as a reservoir for VLDL needed for egg yolk production (eyVLDL). The amino acid composition of apolipoproteins of serum VLDL and eyVLDL are similar, both having lysine at the N-terminus and tyrosine at the C-terminus (Hillyard *et al.*, 1972).

A further lipoprotein fraction in plasma is the low-density lipoprotein (LDL) which carries a larger proportion of plasma cholesterol than other lipoproteins (Hillyard *et al.*, 1955). Its amino acid composition, including C- and N-terminal amino acids, is similar to VLDL (Hillyard *et al.*, 1972) and it is precipitated by anti-VLDL antibody (Luskey *et al.*, 1974). Based on this evidence, Luskey and his colleagues suggest that plasma lipoproteins of the chicken be divided into two groups with densities above and below 1.063 gm cm^{-3}. Lipoproteins of density less than 1.063 gm cm^{-3} (VLDL and LDL) contain as their major protein component a single antigenic polypeptide with a molecular weight less than 25,000 daltons. Lipoproteins with a density greater than 1.063 gm cm^{-3} are referred to as high-density lipoprotein (HDL). HDL has aspartic acid at the N-terminus and alanine and leucine at the C-terminus (Hillyard *et al.*, 1972). The HDL contains more than 30% protein with the lipid portion held in pockets formed by a folded peptide chain (Butler, 1971).

When the VLDL levels increase in the laying hen and estrogenized rooster there are also dramatic rises in calcium levels associated with either shell formation (Winget and Smith, 1959) or calcium deposition in the egg yolk (Urist *et al.*, 1958). Associated with the high calcium levels is the increase in two specialized high-capacity binding proteins, X_1 (a phosphoprotein) and X_2 (a phospholipid lipoprotein) (Urist *et al.*, 1958). When calcium levels were raised artificially to 100 mg%, X_1 and X_2 (chiefly X_1) bound 79 mg%, the lipoproteins and albumin bound 15 mg% with the remaining 6 mg% of calcium remaining unbound (Urist *et al.*, 1958). The X_1 phosphoprotein may be the serum equivalent of phosvitin (Mecham and Olcott, 1949), a principal phosphoprotein of egg yolk. Phosvitin has a molecular weight of 40,000 daltons and contains 10% phosphorus bound as phosphoserine units (Mok *et al.*, 1961). It is produced by the liver and the ovary after estrogen stimulation (Greengard *et al.*, 1965; Gornall *et al.*, 1971).

The calcium-binding fraction of the laying hen's serum has been attributed to the presence in the serum of another typical egg yolk protein, vitellin (Greenberg *et al.*, 1936). Although there may be some doubt as to the absolute efficiency of chemical separation of vitellin and phosvitin (Mecham and Olcott, 1949), this cannot explain the assumptions made by Winget and Smith (1959) on the importance of vitellin, and not phosvitin, as the major calcium-binding protein of serum.

The X_2 lipoprotein of Urist *et al.* (1958) has been called lipovitellin (Schjeide, 1963) and is a high molecular weight protein (400,000 daltons) which migrates electrophoretically with the α_2-globulins. As pointed out previously it does not appear to be the major Ca^{2+} binding protein so the relation between vitellin and lipovitellin is doubtful. The lipovitellin of egg yolk has been fractionated into three components, referred to as α- and β-lipovitellin and lipovitellenin (Cook, 1961) which simply confuses the issue more. Clearly, there is much confusion about the terminology of lipoproteins in the early literature which was exacerbated by arguments over the purity and methods of analysis of individual components (see Cook, 1961; Schjeide and Urist, 1961). The grouping of lipoproteins according to density does not help this confused state and it will remain so until total fractionation of all components can be achieved.

2. *Iron-Binding Proteins*

Phosvitin binds ferric iron strongly (Taborsky, 1962) and may well contribute to half the organic bound iron found in the serum of the laying hen and the estrogenized nonlayers (Ali and Ramsay, 1968). The other iron-binding component is transferrin and *in vitro* the iron shows no exchange between the two organic forms (Ali and Ramsay, 1974). Ali and Ramsay (1974) suggested that in laying birds, transferrin iron is a precursor of phosphoprotein iron.

Fowl transferrin is a glycoprotein and differs from the egg white protein ovotransferrin (conalbumin) in its carbohydrate prosthetic group. The protein structures are identical (Williams, 1962). Transferrin has most of its carbohydrate in a single unit composed of two residues of mannose, two residues of galactose, three residues of *N*-acetylglucosamine and one or two residues of sialic acid (Williams, 1968). Ovotransferrin lacks galactose and sialic acid. Serum transferrin has a molecular size similar to serum albumin and a molecular weight similar to human transferrin (80,000 daltons) (Torres-Medina *et al.*, 1971). In moving boundary (Marshall and Deutsch, 1951) and starch gel (Williams, 1962) electrophoresis, transferrin was classed as a β-globulin but in cellulose acetate or paper electrophoresis (Torres-

Medina *et al.*, 1971) it moved as a γ-globulin. This illustrates the confusion that may arise when referring to plasma proteins according to their electrophetograms. Similarly, duck serum transferrins were electrophoretically heterogeneous (Richter *et al.*, 1969) when either the pH or buffer varied, 2–4 fractions being present whose mobility differed from the bird's ovotransferrins.

The liver and yolk sac are the major organs for transferrin synthesis (Gitlin and Kitzes, 1967) and the spleen may act as a reservoir (Giurgea, 1974). Transferrin functions to transport iron to the bone marrow (Katz and Jandl, 1964) and to the yolk (Williams, 1962) and plays an important role in the general control of iron metabolism (Fletcher and Huehns, 1968) and antimicrobial defense.

3. *Copper-Binding Proteins*

Ceruloplasmin, a copper-containing α_2-globulin, was discovered in mammals in 1948 (Holmberg and Laurell, 1948). When compared with the majority of mammals, the levels of ceruloplasm in poultry are low (Srivastava and Dwaraknath, 1971). Chick ceruloplasmin has a molecular weight of 158,000 daltons and contains 0.2% copper indicating 5 atoms of copper per mole. Amino acid composition and electrophoretic mobilities are similar to those reported for human ceruloplasmin (Starcher and Hill, 1966). It has the enzymatic properties of an oxidase but the evidence and the likely substrate are uncertain (Morrell *et al.*, 1962). *In vitro*, it catalyzes the oxidation of ferrous iron (Curzon and O'Reilly, 1960), and from this Osaki *et al.* (1966) suggested that it plays a biological role in the transfer of iron from cells to plasma transferrin. Such a role was demonstrated in copper-deficient swine (Ragan *et al.*, 1969) and in fowl stressed with *Escherichia* endotoxin (Butler *et al.*, 1973). The ferroxidase hypothesis has been refuted by Shokeir (1972) who has suggested that ceruloplasmin mediates copper transfer to copper-containing enzymes, notably cytochrome *c* oxidase and tyrosinase. It is possible that a minor function of ceruloplasmin functions as a haptoglobin in birds through which lyzed hemoglobin is transported and eliminated. Evidence is scanty, but an α_2-globulin does bind hemoglobin in the duck (Liang, 1957).

D. Blood Coagulation Proteins

Blood coagulation is a complex reaction resulting in the conversion of soluble fibrinogen to insoluble fibrin and catalyzed by thrombin. Thrombin is not present in the blood but it is produced from its precursor, prothrombin, by the action of thromboplastin. In mammals,

thromboplastin is a component of platelets and many tissues which release thromboplastin when damaged. The currently known factors present in the mammalian serum that are involved in thromboplastin production are given in Table IV. Blood coagulation in fowl was reviewed recently (Archer, 1971) and only a brief survey will be presented here. The blood coagulation times for chickens are quoted as ranging from 10–300 seconds (Bigland and Triantaphyllopoulos, 1961) and it has always been noted that the shortest times were recorded when the bleeding was least efficiently done (Archer, 1970).

The intrinsic blood coagulation system of chickens is poor, probably because no discrete platelets occur in birds but analogous nucleated cells called thrombocytes are found (Didisheim *et al.*, 1959). Thrombocytes have a much slower thromboplastin generation time than mammalian platelets. When homologous brain extract is used to investigate avian coagulation times, consistent times as short as 10–15 seconds are normally recorded (Wartelle, 1957) but with heterologous systems times are longer (Dorn and Müller, 1965). Coagulation in birds obviously depends essentially on extrinsic thromboplastin production brought about by general tissue damage.

Fibrinogen is a high molecular weight (~340,000 daltons) glycoprotein (β-globulin) with a mean concentration in chicken plasma of 346 mg% compared to 250–400 mg% for humans (Bigland and Triantaphyllopoulos, 1961). Chicken fibrinogen is only clotted by chicken thrombin (Didisheim *et al.*, 1959). Much of the chemical analysis of fibrinogen has been conducted on mammalian species, particularly human (Blombäck, 1970). Doolittle *et al.* (1962) demonstrated close structural similarities in the fibrinogen molecule which can be traced from cyclostomes to mammals. The evolutionary changes in the fi-

TABLE IV
BLOOD COAGULATION FACTORS IN MAN

I	Fibrinogen
II	Prothrombin
III	Thromboplastin
IV	Calcium
V	Labile factor
VII	Stable factor
VIII	Antihemophilic factor
IX	Christmas factor
X	Stuart-Prower factor
XI	Plasma Thromboplastin Antecedent
XII	Hageman factor
XIII	Fibrin-Stabilizing factor

brinogen molecule have also been discussed (Blombäck, 1970; Doolittle, 1976). Certainly chicken and human fibrinogens share common antigenic determinants (Menache *et al.*, 1973).

The significance of the carbohydrate moieties of most glycoproteins is unknown and it has been suggested that the "nonessential" carbohydrate plays a supportive role by helping the polypeptide chain to assume the correct three-dimensional structure (Schmid, 1972). Removal of sialic acid from some of the components of the clotting mechanism, although not preventing clotting, changes the kinetics (Schmid, 1972). Differences in the hexosamine content as well as the tryptophan/tyrosine ratio between chicken and human fibrinogen have been reported by Guimbault *et al.* (1972) and in the latter case the differences were attributed to variable coagulability. The implication is that differences in avian and mammalian fibrinogen may relate to both peptide as well as carbohydrate units. Fibrinogen is produced by liver cells, probably under the influence of steroid hormones (Pindyck *et al.*, 1975).

Among the other factors concerned with avian blood coagulation (Table IV), prothrombin and thrombin are probably similar to the mammalian forms because chicken thrombin will clot mammalian fibrinogens (Didisheim *et al.*, 1959). Factor IX, the plasma thromboplastin component, (Griminger, 1965) and factor XII (Didisheim *et al.*, 1959) have been reported as absent in fowl, factor XI was assumed absent (Archer, 1971), factor VII present in low concentration (Stopforth, 1970), and factor V present only in low levels (Didisheim *et al.*, 1959). According to Wartelle (1957) all factors reported absent are present in very small quantities.

E. Fibrinolysis in Birds

The physiological converse of coagulation is fibrinolysis. In mammals the proteolytic enzyme (plasmin) which breaks down the fibrin clot is triggered by factor XII (Iatridis and Ferguson, 1961). Such a mechanism protects the animal from a buildup of fibrin deposits in the blood vessels. Because plasmin is an anticoagulant, it is present in the blood as an inactive precursor plasminogen. Fibrinolytic activity has not been produced experimentally in birds by any accepted plasminogen activators, but spontaneous lysis has been observed in the blood of some birds (Hawkey, 1970). The saliva of a bird-feeding bat (*Diaemus youngi*) will activate clot lysis, whereas the saliva of the common Vampire Bat (*Desmodus rotundus*) did not (Hawkey, 1970). Such results indicate the presence in avian blood of a plasminogen and low levels of activators.

F. The Immune Plasma Proteins

A complete discussion of the avian immune response is not possible in this chapter not only because of its complexity but also because immunity in an animal relies on both cell-mediated and humoral responses and as such the cell-mediated response is outside the scope of this review.

Immunity may be divided into innate or natural immunity (not acquired through contact with the antigen or infectious agent) and acquired immunity. Acquired immunity may similarly be divided into passive immunity (administration of antibodies manufactured in another individual) and active immunity (antibodies manufactured by the individual itself on being challenged with an antigen). The identification of whether a particular process is innate or passively acquired presents problems mainly because during an embryo's development antimicrobial agents pass into it from the maternal supply. Although the following section is titled passive immunity, it will also discuss natural agents.

1. Passive Immunity

During the development of the avian embryo an extra embryonic membrane encloses the yolk to become the yolk sac. Antibodies are secreted into this sac by specialized cells of the epithelial lining of the oviduct. The levels of these antibodies transmitted to the embryo increases from day 11 of incubation (Buxton, 1952). At the time of hatching, yolk sac and residual yolk is ingested and γ (IgM) antibodies appear in the circulation (Solomon, 1968a).

Newly hatched chickens are resistant to fowl plague and Newcastle disease because of the presence of high levels of maternal antibody (Solomon, 1971). The presence of such antibodies appears to have the secondary effect of suppressing active immunity (Hallaner, 1936). In the case of "fowl typhoid" produced by *Salmonella gallinarium*, there are agglutinins to the bacterium in the embryonic circulation from day 11 of incubation, but they do not appear to be protective and infected 1-day-old chicks die (Solomon, 1971). The presence of natural opsonins in the embryonic circulation plays only a minor role in combating this infection (Solomon, 1968b) and it is only during the first few days posthatch that there is an acquired resistance to the bacterium (Shaffer *et al.*, 1964). The presence of opsonins facilitating phagocytosis of *S. gallinarium* and not being effective implies either a low population of phagocytes or some deficiency in functional ability. With diseases such as pneumococcal infection, where protection is associated with

humoral antibody, opsonins play an important role (Wright, 1927). Opsonins are generally specific antibodies of the IgM or IgG type.

Innate agglutinins for xenogenetic erythrocytes of different species appear in chick serum at different times after hatching. Erythrocytes of rat and rabbit were agglutinated with serum from 16-day-old chicks and guinea pig with serum from 30-day-old chicks (Bailey, 1923).

2. *Complement*

As with other work discussed in this section, mammalian complement has been investigated in considerable detail, particularly human (Müller-Eberhard, 1975). Complement is the general term, in humans, for eleven serum proteins that participate with antigen–antibody complexes in cytolysis, release of histamine, enhancement of phagocytosis, and capillary permeability changes. Complements are glycoproteins of molecular weight 79,000–400,000 daltons, which migrate electrophoretically with all classes of globulins and are designated C1 to C9. C1 is subdivided into C1q, C1r, and C1s. Avian complement and complement components have been little studied because of titration difficulties. Like the mammalian form, avian complement is calcium- and magnesium-dependent for activation (Wirtz, 1967). Sherman (1919) detected complement in 17-day-old embryos, but it was uncertain whether their presence was autogenic or of maternal origin (Solomon, 1971). Chicken C1 has been detected in the serum of normal and hypogammaglobulinemic animals and, in the latter case, correlated with susceptibility to disease (Gabrielson *et al.*, 1974).

3. *Acquired Immunity*

a. Interferon. These cellular proteins are produced in response to invading viruses, circulate in the blood and are taken up by other body cells to offer general body protection. They are species-specific but lack viral specificity, are nondialyzable, nonsedimentable at 105,000 *g* for 2 hours, and are destroyed by proteolytic enzymes, but are stable to acid (pH 2.0) (Kleinschmidt, 1972). They are produced in chick embryos as early as day 6 of incubation (Isaacs and Barm, 1960) and increase by a factor of 20 times by day 11 of incubation.

Attempts have been made to purify chick interferon (Fautes and Furminger, 1967) but there is still some doubt as to whether the carbohydrate found in the molecule is a functional requirement of antiviral activity or an impurity. Certainly, the amino, disulfide and the γ-S-methyl of methionine are necessary for activity (Kleinschmidt, 1972).

b. Immunoglobulins. The immune system of birds, in common with all vertebrate species, is characterized by the capacity to synthesize humoral antibodies in response to a challenge with antigen. There are five classes of immunoglobulins in humans IgG, IgM, IgA, IgE, and IgD; IgG is the major component. Whether the same components are present in birds is still speculative. Using starch gel electrophoresis and Sephadex gel filtration, the patterns obtained for IgG for several mammalian species were similar, but chicken IgG showed slower mobilities (Mehta and Tomasi, 1969). The IgM of all species examined were extremely variable.

When challenged with infectious bronchitis, chickens show an increase in serum complement fixation titers at 6 days postinoculation and high titers for 12–42 days (Marquardt, 1974). The antibodies were generally associated with the γ-globulin fraction in electrophoretic analysis and are referred to as immunoglobulins (Ig). Chicken, challenged with the parasite *Eimeria tenella* (Mukkur, 1969) or suffering from spirochetosis (Perk and Hart, 1966), showed significant drops in albumin fractions and increased levels of globulins, in particular β-globulin.

The response to antigen challenge is an inherited dominant trait in the case of inbred lines of chickens (Balcarova *et al.*, 1974) and is age dependent (Wolfe *et al.*, 1957). It is poorest soon after hatching and increases through the first 4 weeks, with a sudden increase thereafter.

Based on ultracentrifugal evidence, two immunoglobulins have been separated in chickens (Benedict, 1967), a high molecular weight form (HMW) with a sedimentation constant of 19 S and a low molecular weight form (LMW) of 7.8 S. In duck serum, an immunoglobulin of 5.7 S was found which was not a degradation product of the 7.8 S form and was reported present in a number of divergent vertebrates (Zimmerman *et al.*, 1971). The LMW Ig of chicken and the IgG of man possess several characteristics in common, both representing the predominant Ig class in serum (Walsh *et al.*, 1968). Before discussing their characteristics it will be necessary to outline briefly the chemical structure of the immunoglobulin molecule.

The molecular organization of Ig has been discussed in many excellent reviews (e.g., Edelman and Gall, 1969; Fleischman, 1966). The basic molecule (Fig. 1) is composed of two light (L) chains of molecular weight 22,500 and two heavy (H) chains of molecular weight 50,000–75,000 linked by covalent and disulfide bridges to form a T- or Y-shaped structure.

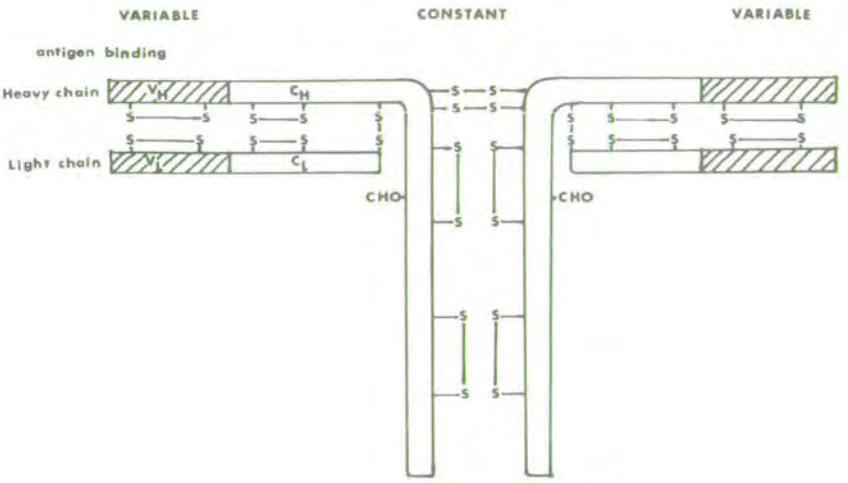

FIG. 1. The structure of immunoglobulin G, after Edelman and Gally (1971).

The L chains contain approximately 217 amino acids and are divided into two groups, κ and λ, distinguished by their amino acid sequences. The relative proportions of the two varies among species. Each light chain is divided into two approximately equal parts, the V chain with an amino terminal unit and the C chain with a carboxyl terminal unit.

The H chains contain 450–576 amino acids depending on Ig class, and are divided into V regions that are variant and C regions that are invariant within a class. In mammals (humans) there are five classes of heavy chains corresponding to the five Ig classes α (IgA), γ (IgG), μ (IgM), δ (IgD), ϵ (IgE). It is the C regions of the heavy chain that determine the Ig class.

Peptide mapping analysis of chicken and human heavy chains demonstrates nine peptides in the two species with identical mobilities (Walsh *et al.*, 1968). Amino acid studies of the peptides, however, show that structurally they are different (Sanders *et al.*, 1973) (Table V). The terms euglobulin and pseudoglobulin are not electrophoretic terms but are based upon sodium sulfate precipitation techniques (Cohn, 1945). The electrophoretic pattern usually remains unchanged after precipitation of the euglobulins (13.5% sodium sulfate). In removal of both the euglobulins and pseudoglobulins (17.4% sodium sulfate) approximately 50% of α-globulins and 25% of the β-globulins are eliminated but none of the γ-globulins.

TABLE V
COMPARISON OF LOW MOLECULAR WEIGHT IMMUNOGLOBULIN HEAVY CHAIN OF CHICKEN WITH γ-CHAIN (IgG) OF MAN

	Chicken	Man
Molecular weight	58,000	48,000
Carbohydrate content	3%, 12%[a]	3%
Number of cysteines	13	10–11
Lysine/arginine ratio	0.6	2.0

[a] Values for pseudoglobulin and euglobulin, respectively (see text).

It would appear that the structural homology between the IMW Ig and IgG first considered by Walsh *et al.* (1968) is not apparent (Table V). Leslie and Clem (1969) have suggested that the avian LMW Ig be referred to as IgY, signifying the fact that it does not resemble any human Ig classes. Sedimentation equilibria studies on LMW Ig indicate a molecular weight of approximately 170,000 daltons, suggesting a structure composed of two H and two L chains. Based on physicochemical, immunochemical, and proteolysis investigations, Tenenhouse and Deutsch (1966) suggested that LMW Ig was homologous to mammalian IgA, a point of view challenged by Lebacq-Verheyden *et al.* (1972) who found a third Ig of γ mobility not related to LMW Ig and referred to as IgA to suggest an IgA homology. This IgA homology was confirmed by Orlans and Rose (1972) and Bienenstock *et al.* (1973) who found that IgA exists in serum in polymeric form (molecular weight of 350,000–360,000 daltons) and monomeric form (170,000 daltons). It was found in and isolated from bile (Lebacq-Verheyden *et al.*, 1972). Because the secretion versus serum ratio for IgA was higher than that for IgG (Lebacq-Verheyden *et al.*, 1974) it confirms the fact that chickens have a secretory immunologic system similar to that found in man.

The HMW Ig of chicken, in terms of gross architecture, resembles the mammalian IgM class (Leslie and Clem, 1969). It has a molecular weight of approximately 900,000 daltons. The H and L chains account for 75 and 25%, respectively, of the mass of the molecule. It is now generally accepted that there are three definite immunoglobulins in avian blood designated IgY, IgA, and IgM.

The secretory immunologic system of birds is probably under the control of the Bursa of Fabricius (Leslie and Martin, 1973) and the IgA-forming cells are derived from cells formerly producing IgM. Certainly the Bursa is the first site of IgM synthesis which occurs in 18-day-old embryos (Thorbecke *et al.*, 1968) when the organ provides

cells which give rise to antibody-producing clones. Animals lacking a Bursa cannot respond to antigens by antibody production even though their thymus is morphologically normal. Thymus cells are evidently involved in the homograph rejection mechanism and hence function similarly to the mammalian thymus (Warner *et al.*, 1962). The mammalian equivalent of the Bursa is uncertain although the human tonsils and Peyer's patches fit the necessary criteria (Cooper *et al.*, 1966). The thymus gland does not produce IgM but after 2 weeks IgY is found in the gland, but probably not in cells of thymic origin. Thorbecke *et al.* (1968) suggested that stem cells from either the bone marrow or the yolk enter the embryonic Bursa as well as other lymphoid tissue on day 14–16 of incubation. Bursal cells then synthesize a hormone that initiates early immunoglobulin synthesis in the organ. The other lymphoid tissues are stimulated to synthesize antibody later by either release of the free hormone or cell types.

III. Egg White Proteins

A. General Comment

Although the components of the avian egg have attracted the attention of workers in all branches of science, there has been a trend since the initial, descriptive investigations (Romanoff and Romanoff, 1949) for the studies to be concerned with fundamental problems of protein chemistry, molecular biology, genetics, etc. Thus, we have a paltry understanding of the overall contributions of the various protein components to the function of the embryo, but a large literature on their occurrence (Sibley, 1960, 1970; Sibley and Ahlquist, 1972), chemical and biological properties (Feeney and Allinson, 1969; Osuga and Feeney, 1974), and phylogenetic traits (Baker, 1970; Manwell and Baker, 1970). There are many admirable reviews and texts dealing with the findings of the protein chemist and their application to phylogenetic studies. This section will be concerned with a discussion of the possible functional roles of the proteins in the embryo.

B. The Role of the Cleidoic Egg

The pivotal role (Freeman and Vince, 1974) of the cleidoic egg in the breeding biology of birds was taken for granted until the demonstration (Ratcliffe, 1970) that the agricultural use of certain pesticides was associated with a reduction in hatchability. Through being weakened, the shell, particularly those of the eggs of raptors, could no longer resist the stresses to which it was exposed in the next. Those

whose concern it is to hatch thousands of domestic chicks per day have long been aware of the contribution of egg traits (size and shape of egg, shell characters, albumen quality) to hatchability (Landauer, 1967) even when the performance of the incubator had been optimized by trial and error (Lundy, 1969). In studies of the natural history of incubation (Drent, 1975; White and Kenney, 1974) the emphasis was largely on behavioral adaptations whereby the parent(s) impose an environment within the nest so that the needs of the developing embryo were met. In an ecological context Lack (1968) sought an interpretation of the means whereby birds achieve population stability. He recognized "problems concerned with eggs" even though the discussion was limited to cryptic markings of the shell and the probable need for the eggshells of waterfowl to be waterproofed.

In discussing the egg and the environment, Needham (1950) noted a complex interplay of factors between the embryo and the environment—a concept that, as stressed by Vince (1973), has been largely ignored by those studying the embryo's behavior. Thus with the avian egg, its sole demand on the abiotic components of the environment is for oxygen, the parent(s) providing heat—or shade, movement and, perhaps, some control over relative humidity. In accepting that the egg's release from a requirement for exogenous water is the last fundamental step in the evolution toward minimum dependence on the external environment, zoologists accepted that the embryo's physiology would have had to have adapted to life in a closed system, and much effort has been directed at topics such as nitrogen metabolism leading to the end product, uric acid, and the acid–base relationship within the egg (Dawes, 1975; see also Freeman, Chapter 3, this volume).

Although achieving independence from exogenous water, the avian egg has become vulnerable to that water. With the high metabolic rate obtained with incubation at 35°–40°C, the embryo, especially in the few days preceding pipping, has a large requirement for O_2 (Freeman and Vince, 1974) that can be satisfied only by diffusion (Wangensteen and Rahn, 1970–1971) across pores (Board *et al.*, 1977) in the calcitic shell. The complement of pores provides a diffusive capacity which matches the demand of the embryo in the last day or so of incubation (Tullett and Board, 1976). It is recognized that the pores provide portals also for the outward diffusion of water vapor (Rahn and Ar, 1974). It is generally accepted that the egg contains at oviposition sufficient water to compensate for loss of up to 18% of egg weight (Freeman and Vince, 1974). In theory the diameter of the pores is such that free water could be drawn in by capillarity and it was surmised

(Board and Halls, 1973a,b) that this could lead to asphyxiation of the embryo, a situation analogous to that following the flooding of an insect's plastron (Hinton, 1968). Board (1974, 1975) demonstrated that the eggshell was water repellent, waterproofed, or both and that work needs to be done to overcome these resistances. When work is done, there is a marked tendency for the eggs to rot during storage (Haines and Moran, 1940).

The problems which confront the egg tend to be ignored in many of the discussions of strategies of egg production (Price, 1974) even though it was noted often that egg production was adapted to counter the relative hostile nature of the environment colonized by the parents. Actually both the parents and the eggs must counter such hostility. When the proposed strategy of adaptation had as its basis the allocation of resources then the concept is germane to this section. Given that there is a limited amount of energy available for reproduction, then this must be allocated optimally to eggs, avoidance of predation, and competitive ability. Predation tends to be associated popularly with food chains; it ought to be considered also in the sense of "energy wastage" by heterotrophic bacteria. If this is accepted, then two questions may be posed. First, have the selective pressures which favor the evolution of a few large eggs—and thus a relatively large allocation of energy—favored also a defense whereby the energy and developing embryo are protected from infection with heterotrophic bacteria? Second, have these pressures favored also a defense which operates in the neonate during both the time that it is acquiring immunological competence and during the period that it takes for the gut and mucous membranes to be colonized by nonpathogenic microorganisms? Those trained in classical immunology (Solomon, 1971) would brook no argument about the neonate's complement of maternal antibodies and they cite examples which provide a clear indication of a relationship between the immunobiological status of parent and offspring. In this context the avian embryo is analogous to those of guinea pigs, man, etc. (Brambell, 1958) in being dependent upon the transmission of antibodies within the parent's body. Nevertheless, human milk is rich in antibodies and other antimicrobial substances that create an environment in the gut which holds the enteropathogens in check while the commensal flora becomes established. Apart from its large content of antibodies, mammalian milk contains many components of the defense system which Tokin (1964) and Board and Fuller (1974) have advanced to account for the defense of the avian embryo and its food reserves. Although having teleological overtones, their hypothesis does permit a novel discussion of the egg white

proteins without requiring excessive cataloging of the achievement of the protein chemists and molecular biologists. It also directs attention to a form of defense (Glynn, 1972) that has been often neglected by those trained in classical immunology. It may also provide another vantage point for discussion of the apparent evolutionary conservatism in birds in general (Prager *et al.*, 1974). Overall, it may reflect the outcome of a situation where selective pressures operate on two independent but interdependent units, the parent and its egg.

C. The Albumen

The albumen (white) occupies the space between the yolk (Fig. 2), bounded by the chalaziferous layer and four-layered vitelline membrane (Bellairs *et al.*, 1963), and the shell membranes which consist of anastomizing fibers with a protein core and a mantle rich in polysaccharides (Cooke and Balch, 1970). In the eggs of ten altricial species discussed by Romanoff and Romanoff (1949), the albumen makes a relatively larger contribution than in precocial species (Table VI). The albumen is synthesized and deposited in the oviduct (Gilbert, 1971) by mechanisms controlled by steroids (Palmiter and Smith, 1973; Rosen *et al.*, 1975; Tuohimaa and Söderling, 1976; Sharma *et al.*,

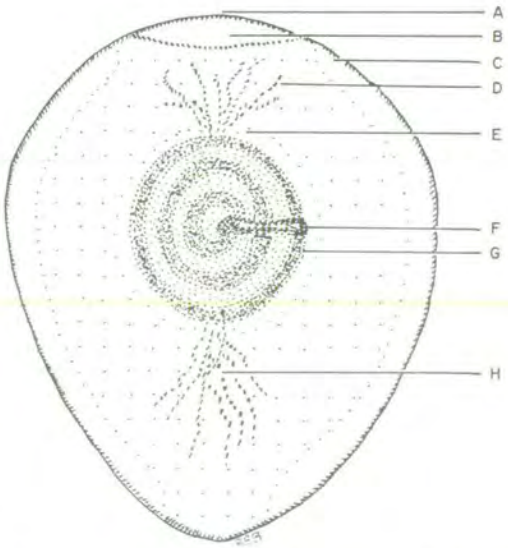

Fig. 2. Structure of egg as seen in longitudinal section. A, shell and shell membranes; B, air cell; C, outer thin white; D, albuminous sac; E, inner thin white; F, blastoderm; G, yolk; and H, chalaza.

TABLE VI
GROSS COMPOSITION OF EGGS

Component (wt %)	Precocial (domestic hen)	Altricial
Albumen[a]	56	61–78.6
Yolk	33	—
Shell/membranes	11	11

[a] Albumen of typical hen's egg (wt %): water (88.5), protein, (10.5), carbohydrate, (0.5), inorganic, [0.5–Na^+, 1.42 (1.27–1.83); K^+, 1.40 (0.94–1.77); Ca^{2+}, 0.13 (0.07–0.29; Mg^{2+}, 0.1; Fe^{3+}, 0.0001; S, 1.99; Cl^-, 1.27; PO_4^-, 0.11]; vitamins (trace).

1976). Amino acids from the blood are extracted in the magnam at a rate corresponding to the rate of albumen synthesis (Edwards *et al.*, 1976). The general composition of the albumen of the domestic hen is given in Table VI.

There are three layers of albumen in freshly laid eggs, each containing different amounts of dissolved material. The approximate percentage of total egg white : dry matter for hen's egg are 23 : 21% in the outer thin white, 57 : 12% in the albuminous sac, and 17 : 13% in inner thin white (Brooks and Taylor, 1955). The protein content of thin and thick white is similar but thick white contains about four times more ovomucin. The thick white in the hen's egg is a weak gel interpenetrated by a system of microscopic elastic fibers (Brooks and Hale, 1959). With storage at ambient temperature several changes occur in the hen's egg. The pH drifts from about 7.4 to 9.5 as CO_2 diffuses across the shell. The amount of outer thin white increases at the expense of the albuminous sac and eventually the inner thin white is transferred across the albuminous sac. The albuminous sac loses its gel state and the density of the white increases as it loses water by evaporation and absorption by the yolk. Finally, the freedom of movement of the yolk increases, due to decay of the albuminous sac, and it rises to make contact with the shell membranes. During the first 5–8 days of incubation much of the water in the albumen is transferred to the subembryonic fluid (Romanoff, 1967; New, 1956). This is associated with a reduction of the albumen's content of Na^+, and Tullett and Board (1976) concluded that a Na^+ pump was involved. There is a close association also between the albumen water loss and a diminution in the resistance of the shell membranes to gaseous diffusion (Tullett and Board, 1976), possibly as a result of the membrane becoming "dry" (Kutchai and Steen, 1971). On about the twelfth day of embryo

development of the domestic hen, the albumen begins to pass via the seroamniotic connection to the amnion and eventually to the gut and lungs of the embryo (Witschi, 1956). There is a migration of ovomucoid from the albumen to the amnion from which it is swallowed and transported via the gut to the yolk sac where it is catabolized by proteases and glycosidases present in the yolk sac membrane (Oegema and Jourdian, 1974a,b). Ovomucoid is present in the embryo's serum on day 12 and in that of the neonate on the seventh day following hatching.

D. The Role of the Albumen and Antimicrobial Defense

The contributions made by the major components of the egg to the maintenance of the embryo (Table VII) emphasize the dependence on the concerted action of many functions of each component. Thus the vulnerable food store of the egg, the yolk, is protected from infection with microorganisms of extragenital origin by the chemical defenses of the albumen and the physical defenses of the shell. Moreover, as noted later, the central location of the yolk, and thus the biological structure of the egg, contributes to its defense.

The egg's vulnerability to addling by heterotrophic microorganisms has attracted insufficient attention. This is due partly to ornithologists assigning infertile eggs, dead embryos, and rotten eggs to the category, addled, and partly to the freedom from contamination which experimental embryologists have enjoyed with their rather casual approach to asepsis during experiments on incubating eggs. However, addling has long been recognized by those who deal with commercial eggs or by those who operate incubators which contain many thousands of eggs. The literature generated from the latter sources has been reviewed elsewhere (Board, 1966, 1968, 1969) and the principal findings can be summarized briefly. First, the majority of eggs of the domestic hen are free from microbial infection at oviposition (Brooks and Taylor, 1955; Harry, 1963a,b) but certain pathogens (*Salmonella*, mycoplasma and viruses) can be transmitted via the yolk and white and cause disease in the newly hatched chick (Payne, 1968; Harry and McClintock, 1972). Second, rot-producing bacteria which contaminate the shell have to be translocated along water-filled pores and be lodged in the shell membranes before the egg contents are at risk (Williams and Whittemore, 1967; Board and Halls, 1973a). Following translocation, there are three possible outcomes: (1) the organisms migrate through the albumen and colonize the surface of the yolk (Sharp and Whitaker, 1927); (2) the infection is confined to the shell membranes until the yolk contacts the shell membranes (Board, 1964); or (3) the bacteria

TABLE VII
THE CONTRIBUTIONS OF COMPONENTS OF THE EGG TO THE MAINTENANCE AND DEVELOPMENT OF THE EMBRYO

Component	Physiological function	Physical protection	Antimicrobial defense
The integument (preening oils, cuticle/cover, true shell and shell membranes[a])	Exchange of respiratory gases Conservation of water Reservoir of Ca^{2+} Depot of carbonate Insulation Conductance of sound waves and photostimuli	Protection from crushing Prevention of water logging Camouflage	Barrier to microbial invasion
Air cell	Air reservoir and induction of breathing Water conservation—a "cold nose" phenomenon	Compensation for changes in pressure	—
Albumen	Reservoir of water Depot of cations (Na^+, Mg^{2+}, K^+) Exchange of O_2, CO_2, H^+ with young embryo Source of protein	Cushioning against damage due to sudden movement Lag against temperature change Scaffolding for yolk and embryo	Viscosity an impediment to bacterial movement Controls rate and extent of microbial growth Passive immunity of chick
Yolk	Principal depot of all major and minor nutrients	Location of young embryo at least distance from heat of brood patches	Passive immunity of chick

[a] Board *et al.* (1977).

pass across the albumen, infect the yolk, and through modifying the food reserves, cause intoxication or starvation of the neonate (Harry, 1957). Of these, point (2) is a common feature of infertile eggs or those stored at subincubation temperatures (Board, 1969) and is probably the one which operates in nature during the laying of a clutch of eggs. Option (3) is a feature of commercial hatcheries if the hygiene is poor. The apparent failure of microorganisms to colonize and grow in the albumen is a feature common to all three. The inadequacy of the

albumen as a medium for microbial growth was implicit in the observations of Gayon (1873), and the tendency in the past century has been to attempt to account for this in terms of the albumen's content of proteins having specific biological properties (Table I). The uniqueness of these properties relative to those of the components of the cellular and humoral defense systems has attracted little comment even though they may represent an extremely old (primitive?) form of defense. It operates without energy consumption, "training," neural or hormonal control in a system lacking a vascular network and it can act against one cell type (the prokaryotic heterotroph) while providing the physiochemical environment required by the eukaryotic cell of the avian embryo (Tokin, 1964; Board and Fuller, 1974).

E. Egg White Proteins

Information about the complement of proteins in the albumen of avian eggs has been a product of innovation in the methods used to isolate, purify, and characterize proteins. Thus gel electrophoresis demonstrates upward of 50 or so "bands" and purification can be achieved by affinity or ion-exchange chromatography, ultrafiltration, etc. (Neurath and Hill, 1975). In practice, most information pertains to the ten major proteins of the albumen of the hen's egg (Osuga and Feeney, 1974) many of which occur widely in bird eggs (Feeney and Allinson, 1969). Since the studies of McCabe and Deutsch (1952) and their assertion that "an egg contains more of its incipient phylogeny than the more superficial aspects of the bird's adult morphology," there have been many phylogenetic studies (Sibley, 1960, 1970; Sibley and Ahlquist, 1972), some of which have established clearly the relatedness of particular birds (Baker and Manwell, 1976) and the recognition that protein polymorphism is widespread (Ferguson, 1971). Support for the assertion "the selection for factors affecting egg white proteins is probably slow, indirect and less drastic" (McCabe and Deutsch, 1952) has come from immunological studies. Prager *et al.* (1974) surmise that slow evolution in some egg white proteins may be associated with the birds' high body temperature or low DNA content may need to be considered alongside our speculation (Section III,B) that selection pressures operate on two independent but interdependent units, the chicken and egg. Detailed studies of some of the highly purified proteins have shown differences in amino acid composition and biological properties (discussed in the following sections).

1. Lysozymes and Ovomucins

The lysozymes (muramidases) cleave the $\beta(1\text{–}4)$ glycosidic linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid in the pep-

tidoglycan heteropolymers which confer geometry and resistance to osmotic lysis to prokaryotes. Their contribution to the total egg white ranges from 3–4% in the chicken, other galliformes, and anseriformes (Feeney and Allinson, 1969) to only trace amounts in penguins (Manwell and Baker, 1973).

The amino acid sequence of several avian lysozymes has been elucidated (summarized in Jollès *et al.*, 1976). Differences also exist in their enzymatic properties (Dianoux and Jollès, 1967; Arnheim *et al.*, 1973; Bailey and Geoffrey, 1975), crystallography (Bott and Sarma, 1975), and immunogenicity (Arnheim and Steller, 1970; Jollès *et al.*, 1976). The latter has been used in a survey, based on immunodiffusion techniques, of the distribution of the chicken (lysozyme *c*) and goose (lysozyme *g*) muramidases in the orders of birds (Prager and Wilson, 1974). Lysozyme *c* was demonstrated in two orders (Galliformes and Anseriformes), whereas lysozyme *g* was found in nine (Anseriformes, Struthioniformes, Rheiformes, Apterygiformes, Tinamiformes, Podicipediformes, Sphenisciformes, Casuriformes, Charadriiformes) and both *c* and *g* in some Anseriformes (Black Swan and Canada Goose). The domestic chicken produces both lysozymes *c* and *g*, the former occurring in egg white and both in the polymorphonuclear leukocytes (Hindenburg *et al.*, 1974). The genetic loci that code for both forms of the enzyme may be common to many species but species-specific regulatory mechanisms control whether one or both are expressed during the production of egg white proteins. Jollès *et al.* (1976) compared sequence differences with immunological differences and derived a cladogram of selected taxa based on lysozyme differences.

The lysis of bacteria by egg white was noted by Laschtschenko (1909) but this property did not become widely recognized until similar observations led Fleming (1922) to appreciate the enzymatic nature of the process and to propose the name, lysozyme, for the lytic agent. Since his account "on a remarkable bacteriolytic element found in tissues and secretions," the enzyme has been accorded a cardinal role in the antimicrobial defense of the egg. Manwell and Baker (1973) discussed the concept that large amounts of lysozyme in egg white may reflect selective pressures associated with eggs being challenged with a large load of microorganisms. Arnheim *et al.* (1973) pondered a possible correlation between the type of muramidase and the dominant prokaryotic type to which eggs are exposed. However, nothing is known about the level and type of bacterial infection of nests, so neither hypothesis can be considered further at the present. In fact, it is not possible to present evidence to support the general view that lysozymes, as enzymes, are important in the egg's defense. Fleming

and those who have followed him (e.g., Garibaldi, 1960) have used a sensitive organism such as *Micrococcus lysodeikticus* in whose cell wall the peptidoglycans are a major component and, as a consequence, many $\beta(1\text{–}4)$ glycosidic bonds are exposed. The addition of lysozyme c to the mixed bacterial flora from eggshells did not reduce the viable count by a significant amount (Board, 1968) and it has been established (Board, 1966, 1969) that gram-negative bacteria are the dominant contaminants of rotten eggs. Such organisms have a relatively small amount of peptidoglycan in their cell envelopes, and additional materials, such as the lipoproteins and lipopolysaccharides, impede the diffusion of lysozyme to its substrate. This may be disrupted experimentally with EDTA at pH 8–7, for example, but whether such disruption occurs in the egg has not been demonstrated. It is notable, even if at the present time anecdotal, that yet another diffusion barrier, a capsule, was a common feature of the gram-negative bacteria isolated from the rotten eggs of waterfowl (Seviour *et al.*, 1972). This may reflect the selectivity imposed by lysozyme *g* which attacks peptidoglycans from gram-negative bacteria more avidly than those from gram-positive ones (Arnheim *et al.*, 1973). If a cardinal role for lysozyme is accepted, particularly with respect to gram-negative bacteria, then it is pertinent to consider the consequences of bacterial lysis. Many of the lysates show great pharmacological activity and may enter the gut, etc. of the embryo. In this case could not the action of lysozyme exacerbate rather than resolve problems? Another, and possibly more fruitful, line of enquiry as to the role of lysozyme had its genesis in commercial problems associated with the breakdown of the albuminous sac and the loss of "quality" of eggs intended for human consumption (Almquist and Lorenz, 1932). Many investigators have concluded that the gel structure of the albuminous sac resulted from a protein–protein interaction and Brooks and Hale (1959) postulated that the mechanical properties of the sac were the product of a network formed from cross-linking of an ovomucin–lysozyme complex. Further, lysozyme will polymerize with itself when the ionic environment is appropriate and reduced soluble ovomucin derivatives can interact with stoichiometric amounts of lysozyme through salt linkages (Robinson and Monsey, 1969a,b). Subsquently, Robinson and Monsey (1971, 1972a,b, 1975) showed that ovomucin of the hen's egg consists of two components: α-ovomucin (MW 210,000) with about 1% (w/w) sialic acid and β-ovomucin [an aggregate (MW 720,000) formed from globular subunits (MW 112, 300)] containing more than 10% (w/w) sialic acid. With incubation at 37°C, thinning of the thick white was associated with degradation of β-ovomucin. Kato *et al.* (1975) endorsed

the concept of an electrostatic attraction between the negative charges of the terminal sialic acid in ovomucin and the positive charges of lysyl ϵ-amino groups in lysozyme. They demonstrated that the extent of interaction of F-ovomucin (their ovomucin fraction rich in sialic acid) and lysozyme was reduced when sialic acid was removed by neuraminidase. It is noteworthy, also, that at the body temperature of the hen, lysozyme has a conformation different from that at 20°C and a diminished affinity for certain substrates (Jollès *et al.*, 1975).

These observations also provide the basis for comparative studies of the rheological properties of egg albumen in general and contribute to another feature of the avian egg, the role of the albuminous sac. It is suggested that the sac performs a scaffolding role which is equal in functional significance to the contribution of the extra embryonic membranes. Its contribution is considered important in two phases of the egg's existence: (1) initially by ensuring that the yolk is retained centrally and thereby protected from colonization by microorganisms (Board, 1964); (2) by ensuring that the embryo which has yet to complete its chorioallantoic membrane is prevented from adhering to the shell membranes, a phenomenon which results in malformation (a "stuck germ"). Both roles are transient. They are performed in the absence of neural or hormonal control and, on completion of their function, they must not be an impediment to the continued development of the embryo. The protein complexes must acquire physical properties which permit their passage across the seroamniotic raphe, and they presumably have to be adapted to the environment existing before the onset of brooding. Thus if the postulated lysozyme–ovomucin complex of the hen's egg were present in the eggs of the ostrich it could be anticipated that, because of damage by exposure for a few minutes to temperatures near 60°C (Garibaldi *et al.*, 1968), it might well be denatured in the time that the Ostrich egg spends on sand at 56°C (Sauer and Sauer, 1966) before the onset of brooding. Dr. S. G. Tullett and I (unpublished) have noted in determinations of the freezing points of albumen that some separate into two phases on thawing whereas others return to a state resembling untreated samples. Such observations imply that some environmental components, especially temperature, may apply strong selective pressures via the egg to the synthetic attributes of the oviduct. Thus a broader biological setting might well aid our understanding of the reasons underlying observations such as the resistance to decay in the albumen of duck and goose eggs (Rhodes and Feeney, 1957); that the albumen of penguin eggs, which contains little lysozyme but large amounts of sialic acid (Feeney *et al.*, 1968; Manwell and Baker, 1973), deteriorates at

about the same rate as that of domestic hens (Feeney and Allinson, 1969); and the correlation between the quality of the thick white and its content of sialic acid (Sauveur, 1975).

2. *Avidin and Ovoflavoprotein*

The basic, tetrameric protein, avidin (Green, 1975), is secreted in the oviducts of reptilia, amphibia and birds, but not mammals, when they are supplied with progestagenic steroids (Tuohimaa, 1975; Tuohimaa and Söderling, 1976). Avidin binds with four moles of biotin (vitamin H), one per subunit, with a dissociation constant of 10^{-5} *M* (Green, 1963a,b, 1964; Green *et al.*, 1971; Chignell *et al.*, 1975). The avidin content in egg white (Feeney and Allinson, 1969) ranges from 0.1 units/gm dried weight of the Herring Gull *(Larus argentatus)* to 16.2 units for the Turkey *(Meleagris gallopavo)*. The ease with which this potent binder can be studied by microbiological assay (Wright and Skeggs, 1944) is probably sufficient evidence to justify the common belief that it can contribute to the defense of the albumen against colonization by microorganisms requiring this vitamin.

Ovoflavoprotein, which can be isolated from the hen's egg white by cellulose ion exchange (Rhodes *et al.*, 1958), and purified by affinity chromatography (Blackenhorn *et al.*, 1975), binds riboflavin (Rhodes *et al.*, 1959) more avidly ($K_a = 7.9 \times 10^8\ M^{-1}$) than the more commonly occurring cofactors of flavoenzymes, FMN ($K_a = 7.3 \times 10^5\ M^{-1}$) or FAD ($K_a < 7 \times 10^4\ M^{-1}$). It is a glycoprotein (32,000 daltons) containing 16 half cystine residues (8 Cyst–Cyst bridges) and consists of two subunits the larger of which contains five of the dithio linkages (Clagett, 1971). The flavoproteins are fairly widespread in birds (Feeney and Allinson, 1969). Homogeneity of precipitin lines was demonstrated by immunodiffusion of the flavoproteins isolated from the yolk, white, and blood serum of domestic hens (Clagett, 1971). By selective breeding of certain strains of domestic hens, the genotypes (RdRd, Rdrd and rdrd) were obtained with the ratio of riboflavin : carrier : protein in the blood stream of 2 : 1 : 0. Hens with the double recessive rapidly excrete riboflavin in the urine and lay eggs which, with little ovoflavoprotein in the albumen, will not support embryo development beyond 10–14 days unless the vitamin (200 mg) is injected through the shell (Buss, 1969; Clagett, 1971).

Although the inhibition of the growth of *Streptococcus pyogenes* and *Lactobacillus casei* has been demonstrated *in vitro* when 10 moles of apoprotein was present for every mole of riboflavin (Rhodes *et al.*, 1959), the half-saturation of the apoprotein in the hen's egg white would give this protein only a minor role in the egg's antimicro-

bial defense (Baker, 1968). This suggestion was based on a consideration of apoprotein in isolation. In practice a riboflavin-requiring microorganism may not be able to glean sufficient vitamin in an environment having many other extremes of environmental parameters (high pH, unavailability of combined nitrogen, Fe^{3+}, avidin, etc).

3. Ovotransferrin

Although the inadequacy of the albumen of the hen's egg as a medium for microbial growth had been recognized for about 100 years, this property was not explained satisfactorily until the studies of Schade and Caroline (1944). They noted that several species of bacteria and a yeast would grow in albumen if Fe^{3+} was added. This led to the demonstration (Alderton *et al.*, 1946) of the iron-binding (2 binding sites per mole; Evans and Holbrook, 1975) properties of conalbumin (ovotransferrin), a protein which is a member of a class of ligands studied extensively in the past 20 years (Crichton, 1975). The transferrins, glycoproteins of ca. 80,000 daltons (Tsao *et al.*, 1974a,b,c), are a common component of bird eggs (Sibley, 1960, 1970), often in polymorphic form (Ferguson, 1971), and occur in a wide range of biological fluids (Feeney and Allinson, 1969). Their evolution can be ascribed presumably to the need for an iron-transport system to operate in situations of pH and oxygen tension where the tendency is for Fe^{3+} to form complex, insoluble hydroxides. In such situations, those cells which require Fe^{3+} have a receptor site whereby transfer of the metal ion from the transferrin is achieved (Crichton, 1975).

In studies with bacteriological media containing known amounts of Fe^{3+} and conalbumin, Theodore and Schade (1965a) noted that the initiation of growth, rate of growth, and total crop of *Staphylococcus aureus* were dependent on the concentration of free iron, which was a function of the percentage iron saturation of the ligand. With albumin *in vitro* deprivation of Fe^{3+} did not cause the death of those organisms which are commonly associated with rotten eggs (Board and Halls, 1973c). It can be inferred, therefore, that a fermentative prokaryote suspended in albumen does not have its metabolism switched from an oxidative to a fermentative type, a phenomenon noted by Theodore and Schade (1965b) when *St. aureus* was deprived of iron by the addition of ovotransferrin to a bacteriological medium which had been depleted of Fe^{3+}. Microorganisms do not retain their viability when suspended in vertebrate serum *in vitro*. Their death was attributed to an interplay of antibody, transferrin, and complement (Fletcher, 1971). From studies of *Pasteurella septica*, Griffiths (1974) concluded that iron deprivation

initiated changes which resulted in the rapid degradation of ribosomal RNA.

The bacteriostatic action of ovotransferrin can be negated if another chelate, 8-hydroxyquinoline, is added to a bacteriological medium (Feeney and Nagy, 1952). It is of interest to note that monoferric ovotransferrins are formed in the presence of the chelates, ATP, and nitrilotriacetate (Donovan *et al.*, 1976), whereas Fe_2-ovotransferrin is formed in their absence (Aisen and Leibman, 1968). Owing presumably to the formation of insoluble hydroxides in systems having O_2 in solution at neutral pH, a wide range of prokaryotes have evolved compounds associated with the scavenging of iron (Snow, 1970). Although such compounds can negate the action of ovotransferrin in albumin *in vitro* (Garibaldi, 1970), there is as yet no evidence that they are produced when a microorganism invades the albumen of the egg. As some members of the enterobacterioceae (Wilkins and Lankford, 1970) can grow even when the transferrin of vertebrate serum is unsaturated, the possibility exists that iron-scavenging compounds, enterobactins, may aid the bacterial colonization of the gut of avian neonates.

The conclusion (Board and Fuller, 1974) that ovotransferrin is the principal component of the antimicrobial defense of the hen's egg is supported by studies on the pigeon, *Columba livia* (Frelinger, 1972). The egg albumen produced by females heterozygous for ovotransferrin was more inhibitory to the growth of *Saccharomyces cerevisiae* than was that produced by homozygous birds. Moreover, the hatching success of eggs containing the polymorphs of ovotransferrin was greater than with those laid by homozygous females. This was taken to be an important factor in the maintenance of polymorphism in pigeons at Hardy–Weinberg equilibrium. This equilibrium was found also with *C. livia* in Belfast (Ferguson, 1971) but not with the recently introduced *Streptopelia decaocto*. The egg data imply that the heterozygous birds are better pioneers than homozygous individuals.

4. Ovomucoid and Ovoinhibitor

The inhibition of proteases is a general property of the ovomucoids and ovoinhibitors of the egg whites of many species of birds (Spiro, 1973; Osuga and Feeney, 1974; Osuga *et al.*, 1974; Donovan and Beardslee, 1975). The ovomucoids tend to have a narrow spectrum of activity. Those of chicken, cassowary, ostrich, emu and rhea inhibit mainly bovine trypsin; that of the quail inhibits both human and bovine trypsin, and that of the pheasant inhibits bovine α-chymotrypsin and subtilisin but not trypsin of human or bovine origin. The ovoinhibitors have a broader spectrum of activity. With

chicken ovoinhibitor, for example, bovine trypsin, bovine α-trypsin, subtilisin, and fungal proteinase are inhibited. Although a role in the antimicrobial defense of the egg has been implied (Ayres and Taylor, 1956), there is no direct evidence to support such a view. It was suggested (Board and Fuller, 1974) that these compounds may act as inhibitors of proteases released by cells which lyse within the egg, for example, those spermatozoa that did not take part in fertilization. The suggestion that bovine colostrum inhibitor may protect immunoglobulins from digestion in the alimentary tract directs attention to another role for the ovoinhibitors and ovomucoids of egg white.

5. *Ovalbumin, Ovomacroglobulin and Ovoglycoproteins*

Ovalbumin, the major protein of the albumin and one having no biological property of note, and two derivatives, Plakalbumin (Linderstrom-Lang and Otteson, 1947; Otteson, 1958) and S-ovalbumin (Smith and Back, 1964), are separated electrophoretically from the prealbumins (Baker, 1968; Feeney and Allinson, 1969). Polymorphism occurs in the ovalbumin of some birds (Lush, 1961).

The 2–3 bands obtained by electrophoretic separation of hens' egg white reflect differences in the proteins' content of phosphate (Perlman, 1952). Ovalbumin A_1 has two phosphates per mole, A_2 has one, and A_3 none. This does not represent a protein polymorphism.

Ovomacroglobulin, a little studied protein, is notable for its marked antigenicity. It contains immunodeterminants which are common to the proteins present in a range of egg whites (Baker, 1968; Feeney and Allinson, 1969). No particular biological property has been ascribed to the minor component of egg white, the ovoglycoprotein (Ketterer, 1965; Baker, 1968; Osuga and Feeney, 1974).

6. *Miscellaneous Components*

In addition to the ten major proteins of the albumin, the secretory immunoglobulins, IgA and IgM, are present in the albumen of unembryonated chicken eggs and in washings of the 19-day-old embryo gut (Rose *et al.*, 1974). The occurrence of enzymes other than lysozyme was summarized by Baker (1968).

7. *Changes with Incubation*

Although the resolution of separation by electrophoresis is impaired by incubation (Csuka *et al.*, 1973), the biological properties of ovotransferrin (Annau and Cochrane, 1962) and lysozyme (Cunningham, 1974) can be demonstrated up to days 12–16 of incubation of hens' eggs. It was noted above that the albumen passes via the seroamniotic

raphe to the amnion, and it has been established that proteins of egg white origin, viz., ovotransferrin (Frelinger, 1970), are included in the embryo's blood. Does this give a passive immunity analagous to that provided via the placenta or colostrum in mammals (Brambell, 1958)?

REFERENCES

Aisen, P., and Leibman, A. (1968). *Biochem. Biophys. Res. Commun.* **30**, 407.
Alderton, G., Ward, W. H., and Fevold, H. L. (1946). *Arch. Biochem.* **11**, 9.
Ali, K. E., and Ramsay, W. N. M. (1968). *Biochem. J.* **110**, 36P.
Ali, K. E., and Ramsay, W. N. M. (1974). *J. Exp. Physiol. Cogn. Med. Sci.* **59**, 159.
Almquist, H. J., and Lorenz, F. W. (1932). *U.S. Egg Poult. Mag.* **38**, 20.
Annau, E., and Cochrane, D. (1962). *Nature (London)* **193**, 879.
Archer, R. K. (1970). *Symp. Zool. Soc. (London)* **27**, 121.
Archer, R. K. (1971). *In* "Physiology and Biochemistry of Domestic Fowl" (D. J. Bell and B. M. Freeman, eds.), Vol. 2, p. 897. Academic Press, New York.
Arnheim, N., and Steller, R. (1970). *Arch. Biochem. Biophys.* **141**, 656.
Arnheim, N., Inouye, M., Law, L., and Laudin, A. (1973). *J. Biol. Chem.* **248**, 233.
Asofsky, R., Trnka, Z., and Thorbecke, G. J. (1962). *Proc. Soc. Exp. Biol. Med.* **111**, 497.
Ayres, J. C., and Taylor, B. (1956). *Appl. Microbiol.* **4**, 356.
Bailey, C. E. (1923). *Am. J. Hyg.* **3**, 370.
Bailey, P., and Geoffrey, C. J. (1975). *Biochem. Soc. Trans.* **3**, 1212.
Baker, C. M. A. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 67–108. Oliver & Boyd, Edinburgh.
Baker, C. M. A. (1970). *Adv. Genet.* **15**, 147.
Baker, C. M. A., and Manwell, C. (1976). *Philos. Trans. R. Soc. London.* **275**, 109.
Balcarova, J., Hala, K., and Hraba, T. (1974). *Folia Biol. (Prague)* **19**, 329.
Baxendale, W., Courtenay, J. S., Phillips, A. W., and Zola, H. (1971). *Int. J. Biochem.* **2**, 419.
Bellairs, R., Harkness, W., and Harkness, E. D. (1963). *J. Ultrastruct. Res.* **8**, 339.
Benedict, A. A. (1967). *Methods Immunol. Immunochem.* **1**, 229-232.
Bienenstock, J., Perey, D. Y. E., Gauldie, J., and Underdown, B. J. (1973). *J. Immunol.* **110**, 524.
Bigland, C. H., and Triantaphyllopoulos, D. C. (1961). *Am. J. Physiol.* **200**, 1013.
Blackenhorn, G., Osuga, D. T., Lee, H. S., and Feeney, R. E. (1975). *Biochim. Biophys. Acta* **386**, 470.
Blombäck, B. (1970). *Symp. Zool. Soc. London.* **27**, 167.
Board, R. G. (1964). *J. Appl. Bacteriol.* **27**, 350.
Board, R. G. (1966). *J. Appl. Bacteriol.* **29**, 319.
Board, R. G. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 133. Oliver & Boyd, Edinburgh.
Board, R. G. (1969). *Adv. Appl. Microbiol.* **11**, 245.
Board, R. G. (1974). *Br. Poult. Sci.* **15**, 415.
Board, R. G. (1975). *Br. Poult. Sci.* **16**, 89.
Board, R. G., and Fuller, F. (1974). *Biol. Rev. Cambridge Philos. Soc.* **49**, 15.
Board, R. G., and Halls, N. A. (1973a). *Br. Poult. Sci.* **14**, 69.
Board, R. G., and Halls, N. A. (1973b). *Br. Poult. Sci.* **14**, 311.
Board, R. G., and Halls, N. A. (1973c). *Br. Poult. Sci.* **14**, 357.
Board, R. G., Tullett, S. G., and Perrott, H. R. (1977). *J. Zool. (London)* **182**, 251–265.
Bott, R., and Sarma, R. (1975). *Acta Crystallogr., Sec. A* **31**, 525.
Brambell, F. W. R. (1958). *Biol. Rev. Cambridge Philos. Soc.* **33**, 488.

Brandt, L. W., Smith, H. D., Andrews, A. C., and Clegg, R. E. (1952). *Arch. Biochem. Biophys.* **36**, 11.
Brooks, J., and Hale, H. P. (1959). *Biochim. Biophys. Acta* **32**, 237.
Brooks, J., and Taylor, D. I. (1955). *Rep. Food Invest. Board, London* No. 6a.
Brown, J. R. (1975). *Fed Proc., Fed. Am. Soc. Exp. Biol.* **34**, 591.
Buss, E. G. (1969). *In* "The Fertility and Hatchability of the Hen's Egg" (T. C. Carter and B. M. Freeman, eds.), p. 109. Oliver & Boyd, Edinburgh.
Butler, E. J. (1971). *In* "Physiology and Biochemistry of the Domestic Fowl" (D. J. Bell and B. M. Freeman, eds.), Vol. 2, pp. 934–961. Academic Press, New York.
Butler, E. J., Curtis, M. S., and Watford, M. (1973). *Res. Vet. Sci.* **15**, 267.
Buxton, A. (1952). *J. Gen. Microbiol.* **7**, 268.
Chignell, C. F., Starkweather, D. K., and Sinha, B. K. (1975). *J. Biol. Chem.* **250**, 5622.
Christov, D., and Rashev, Z., (1961). *Sofii Univ. Biol. Geol. Geogr. Fak. God Kni Biol. (Zool).* **56**, 29.
Clagett, C. O. (1971). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **30**, 127.
Cohn, E. J. (1945). *Science* **101**, 51.
Cook, W. H. (1961). *Nature (London)* **190**, 1173.
Cooke, A. S., and Balch, D. A. (1970). *Br. Poult. Sci.* **11**, 345.
Cooper, M. D., Peterson, R. D. A., and Good, R. A. (1966). *In* "Phylogeny of Immunity" (R. T. Smith, P. A. Miescher, and R. A. Good, eds.), pp. 243–252. Univ. of Florida Press, Gainesville.
Crichton, R. R., ed. (1975). "Proteins of Iron Storage and Transport in Biochemistry and Medicine." North-Holland Publ., Amsterdam.
Csuka, J., Novÿi, J., and Jirošovâ, Z. (1973). *Br. Poult. Sci.* **14**, 203.
Cunningham, F. E. (1974). *Poult. Sci.* **53**, 1561.
Curzon, G. and O'Reilly, S. (1960). *Biochem. Biophys. Res. Commun.* **2**, 284.
Dawes, C. M. (1975). *Biol. Rev. Cambridge Philos. Soc.* **50**, 351.
Dianoux, A. C., and Jollès, P. (1967). *Biochim. Biophys. Acta* **133**, 472.
Didisheim, P., Hattori, K., and Lewis J. H. (1959). *J. Lab. Clin. Med.* **53**, 866.
Donovan, J. W., and Beardslee, R. A. (1975). *J. Biol. Chem.* **250**, 1966.
Donovan, J. W., Beardslee, R. A., and Ross, K. D. (1976). *Biochem. J.* **153**, 631.
Doolittle, R. F. (1976). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **35**, 2145.
Doolittle, R. F., Oncley, J. C., and Surgenor, D. M. (1962). *J. Biol. Chem.* **237**, 3123.
Dorn, P., and Müller, F. (1965). *Zentralbl. Veterinaer Med., Reihe A* **12**, 380.
Drent, R. (1975). "Avian Biology" (D. S. Farner and J. R. King, eds.), Vol. V, p. 333. Academic Press, New York.
Edelman, G. M., and Gall, W. E. (1969). *Annu. Rev. Biochem.* **38**, 415.
Edelman, G. M., and Gally, J. (1971). *New Sci.* **52**, 213.
Edwards, N. A., Luttrell, V., and Nir, I. (1976). *Comp. Biochem. Physiol. B* **53**, 183.
Evans, R. W., and Holbrook, J. J. (1975). *Biochem. J.* **145**, 201.
Fautes, K. H., and Furminger, E. G. S. (1967). *Nature (London)* **216**, 71.
Feeney, R. B., and Nagy, D. A. (1952). *J. Bacteriol.* **64**, 629.
Feeney, R. E., and Allison, R. G. (1969). "Evolutionary Biochemistry of Proteins." Wiley (Interscience), New York.
Feeney, R. E., Allison, R. G., Osuga, D. T., Bigler, J. C., and Miller, H. T. (1968). *Antarct. Res. Ser.* **12**, 151.
Ferguson, A. (1971). *Comp. Biochem. Physiol. B* **38**, 477.
Fleming, A. (1922). *Proc. R. Soc. London, Ser. B* **93**, 306.
Fletcher, F. (1971). *Immunology* **20**, 493.
Fletcher, J. C., and Huehns, E. R. (1968). *Nature (London)* **218**, 1211.
Fleischman, J. B. (1966). *Annu. Rev. Biochem.* **35**, 835.

Foster, J. F. (1960). *In* "The Plasma Proteins" (F. W. Putham, ed.), Vol. 1, p. 179. Academic Press, New York.
Fredrickson, D. S., and Gordon, R. S., Jr. (1958). *Physiol. Rev.* **38**, 585.
Freeman, B. M., and Vince, M. A. (1974). "Development of the Avian Embryo." Chapman & Hall, London.
Frelinger, J. A. (1970). *Science* **171**, 1260.
Frelinger, J. A. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69**, 326.
Fried, M., and Chum, P. W. (1971). *Comp. Biochem. Physiol. B* **39**, 523.
Gabrielson, A. E., Linna, T. J., Weite-Kamp, D. P., and Pickering, R. J. (1974). *Immunology* **27**, 463.
Garibaldi, J. A. (1960). *Food Res.* **25**, 337.
Garibaldi, J. A. (1970). *Appl. Microbiol.* **20**, 558.
Garibaldi, J. A., Donovan, J. W., Davis, J. G., and Cimino, S. L. (1968). *J. Food Sci.* **33**, 514.
Gayon, M. U. (1873). *C. R. Hebd. Seances Acad. Sci.* **76**, 232.
Gilbert, A. B. (1971). *In* "Physiology and Biochemistry of the Domestic Fowl" (D. J. Bell and B. M. Freeman, eds.), Vol. 3, p. 1163. Academic Press, New York.
Gitlin, D., and Kitzes, J. (1967). *Biochim. Biophys. Acta* **145**, 334.
Giurgea, R. (1974). *Stud. Univ. Babes-Bolyai, Ser. Biol.* **19**, 137.
Glynn, A. A. (1972). *In* "Microbial Pathogenicity in Man and Animals" (H. Smith and J. H. Pearce, eds.), p. 75. Cambridge Univ. Press, London and New York.
Gomall, D. A., Kuksis, A., Pinteric, L., and Mookerjea, S. (1971). *Can. J. Biochem. Physiol.* **49**, 671.
Green, N. M. (1963a). *Biochem. J.* **89**, 585.
Green, N. M. (1963b). *Biochem. J.* **89**, 609.
Green, N. M. (1964). *Biochem. J.* **92**, 16C–17C.
Green, N. M. (1975). *Adv. Protein Chem.* **29**, 85.
Green, N. M., Konieczyny, L., Toms, E. J., and Valentine, R. C. (1971). *Biochem. J.* **125**, 781.
Greenberg, D. M., Larsan, C. E., Pearson, P. B., and Bumester, B. M. (1936). *Poult. Sci.* **15**, 483.
Greengard, O., Sentenac, A., and Acs, G. (1965). *J. Biol. Chem.* **240**, 1687.
Griffiths, E. (1974). *Biochim. Biophys. Acta* **340**, 400.
Griminger, P. (1965). *In* "Avian Physiology" (P. D. Sturkie, ed.), 2nd ed., pp. 21–25. Cornell Univ. Press (Comstock), Ithaca, New York.
Guimbault, P. R., Szabados, L., and Mester, L. (1972). *C. R. Hebd. Seances Acad. Sci., Ser. D.* **275**, 3013.
Haines, R. B., and Moran, T. (1940). *J. Agric. Sci.* **40**, 453.
Hallaner, C. (1936). *Z. Hyg. Infektionskr.* **118**, 605.
Harry, E. G. (1957). *Vet. Rec.* **69**, 1.
Harry, E. G. (1963a). *Br. Poult. Sci.* **4**, 63.
Harry, E. G. (1963b). *Br. Poult. Sci.* **4**, 91.
Harry, E. G., and McClintock, M. (1972). *In* "Safety in Microbiology" (D. A. Shapton and R. G. Board, eds.), p. 121. Academic Press, New York.
Hawkey, C. M. (1970). *Symp. Zool. Soc. London.* **27**, 133.
Heim, W. G., and Schechtman, A. M. (1954). *J. Biol. Chem.* **209**, 241.
Hillyard, L. A., Entenman, C., Feinberg, H., and Chaikoff, I. L. (1955). *J. Biol. Chem.* **214**, 79.
Hillyard, L. A., Entenman, C., and Chaikoff, I. L. (1956). *J. Biol. Chem.* **223**, 359.
Hillyard, L. A., White, H. M., and Pangburn, S. A. (1972). *Biochemistry.* **11**, 511.

Hindenburg, A., Spitzragel, J., and Aynheim, N. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1974.
Hinton, H. E. (1968). *Adv. Insect Physiol.* **5**, 65.
Holmberg, C. G., and Laurell, C. B. (1948). *Acta Chem. Scand.* **2**, 550.
Hughes, L. (1954). *In* "The Proteins" (H. Neurath and K. Bailey, eds.), 1st ed., Vol. 2, Part B, pp. 663–754. Academic Press, New York.
Iatridis, S. G., and Ferguson, J. H. (1961). *Thromb. Diath. Haemorrh.* **6**, 411.
Isaacs, A., and Barm, S. (1960). *Lancet* **2**, 946.
Jollès, J. F., Schoentgen, F., Jollès, P., Prager, E. M., and Wilson, A. C. (1976). *J. Mol. Evol.* **8**, 59.
Jollès, P., Saint-Blanchard, M., Allary, M., Perrin, J. P., and Cozzone, P. (1975). *FEBS Lett.* **55**, 165.
Karush, F. (1950). *J. Am. Chem. Soc.* **72**, 2705.
Kato, A., Imoto, T., and Tagishita, K. (1975). *Agric. Biol. Chem.* **39**, 541.
Katz, J. H., and Jandl, J. H. (1964). *In* "Iron Metabolism" (F. Gross, ed.), pp. 103–117. Springer-Verlag, Berlin and New York.
Ketterer, B. (1965). *Biochem. J.* **96**, 372.
Kimura, M., Yokoyama, Y., Hashimoto, I., Arakig, T., Sakurazaw, M., Isogai, I., and Mahita, N. (1970). *Jpn. Poult. Sci.* **7**, 144.
Kleinschmidt, W. J. (1972). *Annu. Rev. Biochem.* **41**, 517.
Konyshev, V. A. (1968). *Dokl. Akad. Nauk SSSR* **182**, 477.
Kristjansson, P. K., Taneja, G. C., and Gowe, R. S. (1963). *Br. Poult. Sci.* **4**, 239.
Kutchai, H., and Steen, J. B. (1971). *Respir. Physiol.* **11**, 265.
Lack, D. (1968). "Ecological Adaptations for Breeding in Birds." Methuen, London.
Landauer, W. (1967). *Storrs, Agric. Exp. Stn. Bull* **1** (rev.).
Laschtschenko, P. (1909). *Z. Hyg. Infektions.* **64**, 419.
Lebacq-Verheyden, A. M., Vaerman, J. P., and Heremans, J. F. (1972). *Immunology.* **22**, 165.
Lebacq-Verheyden, A. M., Vaerman, J. P., and Heremans, J. F. (1974). *Immunology.* **27**, 683.
Leslie, G. A., and Clem, L. W. (1969). *J. Exp. Med.* **130**, 1337.
Leslie, G. A., and Martin, L. N. (1973). *J. Immunol.* **110**, 959.
Liang, C. C. (1957). *Biochem. J.* **66**, 552.
Linderstrom-Lang, K., and Ottesen, M. (1947). *Nature, (London)* **159**, 807.
Lundy, H. (1969). *In* "The Fertility and Hatchability of the Hen's Egg" (R. C. Carter and B. M. Freeman, eds.), p. 143. Oliver & Boyd, Edinburgh.
Lush, I. E. (1961). *Nature (London)* **189**, 981.
Lush, I. E. (1963). *Br. Poult. Sci.* **4**, 255.
Luskey, K. L., Brown, M. S., and Goldstein, J. C. (1974). *J. Biol. Chem.* **249**, 5939.
McCabe, R. A., and Deutsch, H. F. (1952). *Auk* **69**, 1.
McIndoe, W. M. (1962). *Nature, (London)* **195**, 353.
Manwell, C., and Baker, C. M. A. (1970). "Molecular Biology and the Origin of Species: Heterosis, Protein Polymorphism and Animal Breeding." Sidgwick & Jackson, London.
Manwell, C., and Baker, C. M. A. (1973). *Ibis* **115**, 586.
Marquardt, W. W. (1974). *Avian Dis.* **18**, 105.
Marshall, M. E., and Deutsch, H. F. (1950). *J. Biol. Chem.* **185**, 155.
Marshall, M. E., and Deutsch, H. F. (1951). *J. Biol. Chem.* **189**, 1.
Mayes, P. (1973). *In* "Review of Physiological Chemistry" (H. A. Harper, ed.), p. 14. Lange Med. Rubl, Los Altos, California.

Mecham, D. K., and Olcott, H. S. (1949). *J. Am. Chem. Soc.* **71**, 3670.
Mehta, P. D., and Tomasi, T. B. (1969). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **28**, 820. Abstr. 3131
Menache, D., Cesbron, N., Guillon, M. C., and Shlegel, N. (1973). *Thromb. Diath. Haemorrh.* **30**, 72.
Mok, C. C., Martin, W. G., and Common, R. H. (1961). *Can. J. Biochem. Physiol.* **39**, 109.
Moore, D. H., Shen, S. C., and Alexander, C. S. (1945). *Proc. Soc. Exp. Biol. Med.* **58**, 307.
Morrell, A. G. Aisen, P., and Scheinberg, I. H. (1962). *J. Biol. Chem.* **237**, 3455.
Mukkur, T. K. S. (1969). *Exp. Parasitol.* **26**, 1.
Müller-Eberhard, H. J. (1975). *Annu. Rev. Biochem.* **38**, 697.
Nace, G. W. (1953). *J. Exp. Zool.* **122**, 423.
Needham, J. (1950). "Biochemistry and Morphogenesis." Cambridge Univ. Press, London and New York.
Neurath, H., and Hill, R. L., eds. (1975). "The Proteins," 3rd ed., Vol. 1. Academic Press, New York.
New, D. A. T. (1956). *J. Embryol. Exp. Morphol.* **4**, 221.
Noble, R. C., and Moore, J. H. (1966). *In* "Physiology of the Domestic Fowl" (C. Horton-Smith and E. C. Amoroso, eds.), p. 87. Oliver & Boyd, Edinburgh.
Noyan, A., Lossow, W. V., Brot, N., and Chaikoff, I. L. (1964). *J. Lipid Res.* **5**, 538.
Oegema, T. R., and Jourdian, G. W. (1974a). *Arch. Biochem. Biophys.* **160**, 26.
Oegema, T. R., and Jourdian, G. W. (1974b). *J. Exp. Zool.* **189**, 147.
Orlans, E., and Rose, M. E. (1972). *Immunochemistry* **9**, 833.
Osaki, S., Johnson, D. A., and Frieden, E. (1966). *J. Biol. Chem.* **241**, 2746.
Osuga, D. T., and Feeney, R. E. (1974). *In* "Toxic Constituents of Animal Foodstuffs" (I. E. Liener, ed.), 2nd., p. 39. Academic Press, New York.
Osuga, D. T., Bigler, J. C., Uy, R. L., Sjöberg, L., and Feeney, R. E. (1974). *Comp. Biochem. Physiol. B* **43**, 519.
Ottesen, M. (1958). *C. R. Trav. Lab. Carlsberg* **30**, 211.
Palmiter, R. D., and Smith, L. T. (1973). *Nature (London), New Biol.* **246**, 74.
Paulov, S. (1972). *Biologia (Bratislava).* **27**, 887.
Payne, L. N. (1968). *In* "Egg Quality: A Study of the Hen's Egg" (T. C. Carter, ed.), p. 181. Oliver & Boyd, Edinburgh.
Perk, K., and Hart, I. (1966). *Avian Dis.* **10**, 208.
Perlman, G. E. (1952). *J. Gen. Physiol.* **25**, 711.
Peters, T., Logan, A. C., and Sanford, C. A. (1958). *Biochim. Biophys. Acta* **30**, 88.
Phelps, C. F., and Antonini, E. (1975). *Biochem. J.* **147**, 385.
Pindyck, J., Mosesson, M. W., Rooni, M. W., and Levere, R. D. (1975). *Biochem. Med.* **21**, 22.
Prager, E. M., and Wilson, A. C. (1974). *J. Biol. Chem.* **249**, 7295.
Prager, E. M., Brush, A. H., Nolan, R. G., Nakanishi, M., and Wilson, A. C. (1974). *J. Mol. Evol.* **3**, 243.
Price, P. W. (1974). *Evolution* **28**, 76.
Quinteros, I.R., Stevens, R. W., Stomont, C., and Asmundson, U.S. (1964). *Genetics* **50**, 579.
Ragan, H. A., Nacht, S., Lee, G. R., Bishop, C. R., and Cartwright, G. E. (1969). *Am. J. Physiol.* **217**, 1320.
Rahn, H., and Ar, A. (1974). *Condor* **76**, 147.
Ratcliffe, D. A. (1970). *J. Appl. Ecol.* **7**, 67.
Rhodes, M. B., and Feeney, R. E. (1957). *Poult. Sci.* **36**, 891.
Rhodes, M. B., Azari, P. R., and Feeney, R. E. (1958). *J. Biol. Chem.* **230**, 399.

Rhodes, M. B., Bennett, N., and Feeney, R. E. (1959). *J. Biol. Chem.* **234**, 2054.
Richter, H., Gurtler, H., Hoffman, G., and Diettrich, M. (1969). *Acta Biol. Med. Ger.* **23**, 863.
Robinson, D. S., and Monsey, J. B. (1969a). *Biochem. J.* **115**, 64P.
Robinson, D. S., and Monsey, J. B. (1969b). *Biochem. J.* **115**, 65P.
Robinson, D. S., and Monsey, J. B. (1971). *Biochem. J.* **121**, 537.
Robinson, D. S., and Monsey, J. B. (1972a). *J. Food Sci. Agric.* **23**, 29.
Robinson, D. S., and Monsey, J. B. (1972b). *J. Food Sci. Agric.* **23**, 893.
Robinson, D. S., and Monsey, J. B. (1975). *Biochem. J.* **147**, 55.
Romanoff, A. L. (1967). "Biochemistry of the Avian Embryo." Wiley, New York.
Romanoff, A. L., and Romanoff, A. J. (1949). "The Avian Egg." Wiley, New York.
Rose, M. E., Orlans, E., and Buttress, N. (1974). *Eur. J. Immunol.* **4**, 521.
Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., and O'Malley, B. W. (1975). *Biochemistry* **14**, 69.
Sanders, B. G., Travis, J. C., and Wiley, K. L. (1973). *Comp. Biochem. Physiol. B* **45**, 189.
Sauer, E. G. F., and Sauer, E. M. (1966). *Living Birds* **5**, 45.
Sauveur, B. (1975). *Ann. Zootech.* **24**, 170.
Schade, A. L., and Caroline, L. (1944). *Science* **100**, 14.
Schjeide, O. A. (1963). *Prog. Chem. Fats Other Lipids* **6**, 253.
Schjeide, O. A., and Urist, M. R. (1956). *Science* **124**, 1242.
Schjeide, O. A., and Urist, M. R. (1961). *Nature (London)* **90**, 1175.
Schmid, K. (1972). *Chimia* **26**, 405.
Seviour, E. M., Sykes, F. R., and Board, R. G. (1972). *Br. Poult. Sci.* **13**, 549.
Shaffer, M. F., Bridges, J. F., Clemmer, D. E., and Pontobbidan, K. C. (1964). *Am. J. Hyg.* **80**, 377.
Shamra, O. P., Borek, E., and Martinez-Hernandez, A. (1976). *Nature (Lond.)* **259**, 588.
Sharp, P. F., and Whitaker, R. (1927). *J. Bacteriol.* **14**, 17.
Sherman, H. W. (1919). *J. Infect. Dis.* **25**, 256.
Shokeir, M. H. K. (1972). *Clin. Biochem.* **5**, 115.
Sibley, C. G. (1960). *Ibis* **102**, 215.
Sibley, C. G. (1970). *Bull. Peabody Mus.* No. 32.
Sibley, C. G., and Ahlquist, J. E. (1972). *Bull. Peabody Mus.* No. 39.
Simkin, P. A. (1972). *Proc. Soc. Exp. Biol. Med.* **139**, 604.
Smith, A. B., and Back, J. F. (1964). *Aust. J. Biol. Sci.* **18**, 365.
Snow, G. A. (1970). *Bacteriol Rev.* **34**, 99.
Solomon, J. B. (1968a). *Immunology* **15**, 197.
Solomon, J. B. (1968b). *Immunology* **15**, 219.
Solomon, J. B. (1971). "Foetal and Neonate Immunity." North-Holland Pub., Amsterdam.
Spiro, R. G. (1973). *Adv. Protein Chem.* **27**, 350.
Starcher, B., and Hill, C. H. (1966). *Biochim. Biophys. Acta* **127**, 400.
Stopforth, A. (1970). *J. Comp. Pathol.* **80**, 525.
Stratil, A. (1970). *Anim. Blood Groups Biochem. Genet.* **1**, 15.
Srivastava, K. B., and Dwaraknath, P. K. (1971). *Indian J. Anim. Sci.* **41**, 1044.
Taborsky, G. (1962). *Biochemistry.* **2**, 266.
Tan, A. T., and Woodworth, R. C. (1968). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **27**, 780.
Tata, J. R., and Shellaburger, C. J. (1959). *Biochem. J.* **72**, 608.
Tenenhouse, H. S., and Deutsch, H. F. (1966). *Immunochemistry* **3**, 11.
Theodore, T. S., and Schade, A. L. (1965a). *J. Gen. Microbiol.* **39**, 75.
Theodore, T. S., and Schade, A. L. (1965). *J. Gen. Microbiol.* **40**, 385.

Thorbecke, G. J., Warner, N. L., Hochwald, G. M., and Ohanian, S. H. (1968). *Immunology.* **15**, 123.
Tokin, B. P. (1964). *Folia Biol. (Prague)* **10**, 61.
Torres-Medina, A., Rhodes, M. B., and Mussman, H. C. (1971). *Poult. Sci.* **50**, 1115.
Tsao, D., Azari, P., and Phillips, J. L. (1974a). *Biochemistry.* **13**, 397.
Tsao, D., Morris, D. H., Azari, P., Tengerdy, R. P., and Phillips, J. L. (1974b). *Biochemistry.* **13**, 403.
Tsao, D., Azari, P. R., and Phillips, J. L. (1974c). *Biochemistry.* **13**, 408.
Tullett, S. G., and Board, R. G. (1976). *Br. Poult. Sci.* **17**, 441.
Tuohimaa, P. (1975). *Histochemie* **44**, 95.
Tuohimaa, P., and Söderling, E. (1976). *Acta Endocrinol. (Copenhagen)* **81**, 593.
Urist, M. R., Schjeide, C. A., and McLean, F. C. (1958). *Endocrinology* **63**, 570.
Vanstone, W. E., Maw, W. A., and Common, R. H. (1955). *Can. J. Biochem. Physiol.* **33**, 891.
Vince, M. A. (1973). *In* "Studies on the Development of Behavior and the Nervous System" (G. Gottlieb, ed.), Vol. 1. Academic Press, New York.
Walsh, J. A., Lisowska-Berstein, B., and Lamm, M. F. (1968). *Biochim. Biophys. Acta* **168**, 67.
Wangensteen, O. D., and Rahn, H. (1970–1971). *Respir. Physiol.* **11**, 31.
Warner, N. L., Szenberg, A., and Burnet, F. A. (1962). *Aust. J. Exp. Biol.* **40**, 373.
Wartelle, O. (1957). *Rev. Hematol.* **12**, 350.
White, F. N., and Kenney, J. L. (1974). *Science* **186**, 107.
Wilkins, T. D., and Lankford, C. E. (1970). *J. Infect. Dis.* **121**, 129.
Williams, J. (1962). *Biochem. J.* **83**, 355.
Williams, J. (1968). *Biochem. J.* **108**, 57.
Williams, J. E., and Whittemore, A. D. (1967). *Avian Dis.* **11**, 467.
Winget, C. M., and Smith, A. H. (1959). *Am. J. Physiol.* **196**, 371.
Wirtz, G. H. (1967). *Immunochemistry.* **4**, 118.
Wise, R. W., Ketterer, B., and Hansen, I. A. (1964). *Comp. Biochem. Physiol.* **12**, 439.
Witschi, E. (1956). "Development of Vertebrates." Saunders, Philadelphia, Pennsylvania.
Wolfe, H. R., Mueller, A., Neess, J., and Tempelis, C. (1957). *J. Immunol.* **9**, 142.
Wright, H. D. (1927). *J. Pathol. Bacteriol.* **30**, 185.
Wright, L. D., and Skeggs, H. R. (1944). *Proc. Soc. Exp. Biol. Med.* **56**, 95.
Zaccheo, D., and Grossi, C. E. (1967). *J. Embryol. Exp. Morphol.* **18**, 289.
Zimmerman, B., Shalatin, N., and Grey, H. M. (1971). *Biochemistry* **10**, 482.

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REVIEW

The Antimicrobial Defense of Avian Eggs: Biological Perspective and Chemical Basis

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TRANTER, H. S., AND BOARD, R. G. The Antimicrobial Defense of Avian Eggs: Biological Perspective and Chemical Basis. *J. Appl. Biochem.* **4**, 295–338 (1982).

The principal food reserve, the yolk, of the avian egg is protected from microbial infection by the interaction of the physical defense afforded by the egg's integument (cuticle, calcite shell, shell membranes, and limiting membrane) and the chemical composition of the albumen. This interplay is dependent upon the biological structure of the egg in that the albuminous sac of a freshly laid egg isolates the yolk from the primary nidus of infection, the shell membranes. Apart from its alkalinity (pH 9–10), the albumen contains lysozyme which hydrolyses the peptidoglycan in the cell walls of eubacteria, and the following glycoproteins (biological activity given in parentheses): ovotransferrin (chelation of metal ions, especially Fe^{3+}); avidin (binding of biotin); apoprotein (binding of riboflavin); ovomucoids (inhibition of trypsin and chymotrypsin); ovoinhibitor (inhibition of bovine trypsin and certain microbial proteases); and a ficin-papain inhibitor. Many studies with Gram-negative bacteria, the principal contaminants of addled eggs, have shown that ovotransferrin is the major component of the defense present in the albumen. Through chelating Fe^{3+}, it prevents microbial growth, its effectiveness being enhanced by the alkalinity of the albumen and the latters small content of simple nitrogenous compounds. The cause of death of iron-deprived microorganisms incubated at the upper limits of their temperature range has not been elucidated. Moreover there is too little evidence in support of the claims that microorganisms can counter the Fe^{3+}-deficient state of the albumen through the production of iron-scavenging compounds such as hydroxamates or catechols. Indeed should enterobactin be produced, it is probable that its chelating potential would be rapidly diminished as a consequence of alkaline hydrolysis. Lysozyme seems to play a minor role in the chemical defense system of avian eggs. It is probable that its principal contribution is to the biological structure of avian eggs: the gelatinous properties of the albuminous sac result in part from an interaction of this enzyme with ovomucin.

INTRODUCTION

Animals that oviposit eggs must evolve a strategy such that population stability is achieved. According to Price (1) there has been a general and long established consensus that stability can be achieved by a strategy based on "balanced mortality"; the production of a large number of eggs per female per breeding season is essential when the probability of survival of an individual is low and vice versa. In other words, the number of eggs produced provides an index of the relative hostility of the environment in which the parents live. An alternative view (2) is that the level of available food is the determinant of fecundity and hence of population size. With its

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emphasis on the availability of resources, this latter strategy is probably near to the heart of the problems encountered by egg-laying animals in that it invokes the argument that available energy may well be the key determinant of fecundity. Indeed Cody (3) adopted this argument in a general theory based upon the principle of allocation when considering the breeding biology of birds. In practice he states that the amount of energy available for reproduction must be allocated optimally to egg production, and the parents' avoidance of predation and maintenance of competitive ability so that they get a fair share of the available food. Critics of this theory (e.g., (1)) have pointed out that parasites, pathogenic microorganisms, and viruses as well as unfavorable nesting sites ought to be considered as important and, at times, critical sinks for the energy available to the parents.

Although predation has been identified as a potential drain on available energy, the predators are normally considered to be members of the food chain in the environment in which a particular species of birds lives, viz., hawks that prey on passerines. Indeed little attention has been given to "micropredators," the heterotrophic bacteria and fungi that would exploit the food store in the egg should they invade the major reserve, the yolk. With birds this would be of particular importance because they have evolved (4) the cleidoic (closed box) egg in which all the materials, including water, essential for embryo development and the maintenance of the chick for a few hours or days post-hatching are present at oviposition. Thus a bird's egg represents an enormous allocation of the energy available for reproduction. An example of the extreme of this situation is provided by the kiwis, the smallest members of the flightless Ratites (5). Thus the 403 g^{-1} egg of the brown kiwi, *Apteryx australis* (body wt, 1.5–3.3 kg), contains a 250 g^{-1} yolk which represents ca. 91% of the total energy (ca. 4600 kJ) of the egg.

Although the evolution of the cleidoic eggs offers the tactical choice, a few large versus many small eggs (and hence chicks), it can only succeed when the large energy reserve in a large egg is protected against colonization by microorganisms. The purpose of this review is to provide a detailed account of the chemical defense systems that have evolved in order that the yolk is protected against infection.

THE MAJOR DEFENSE SYSTEMS

There are three major components of the antimicrobial defense of avian eggs: (i) the physical defense provided by the egg's integument (the cuticle, calcite shell, the two shell membranes, and the limiting membrane—see Fig. 1); (ii) the chemical defense systems present in the albumen; and (iii) the defense based on the biological structure of the egg (Fig. 1).

Physical Defense

The contention (6) that the shell impedes microbial infection of the contents of the hen's egg was based initially on the following evidence: (a) less than 1% of nest-clean eggs rot during storage (7); (b) the levels and incidence of contamination of eggs having cracked shells are greater than those having undamaged ones (8); and (c) the rate and incidence of rotting can be increased merely by cracking eggs before exposing them to rot-producing bacteria (9). The normal shell of the domestic hen contains 7000–17,000 pores (10) with diameters in the range 9–35 μm (11, 12), a size that would not be expected to hinder the movement of bacterial cells.

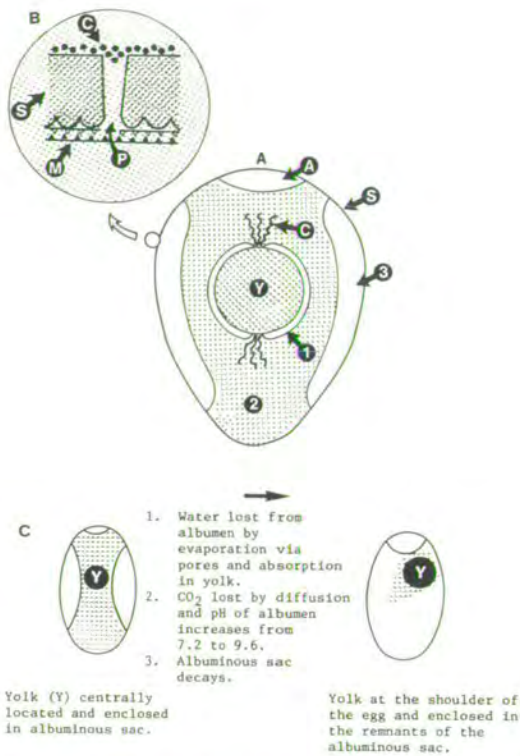

FIG. 1. The structure of the hen's egg. (A) Longitudinal section: A, air cell; C, chalaza; S, shell; and Y, yolk. The white of freshly laid egg consists of the inner (1) and outer thin (3) white and the albuminous sac (2). (B) Radial section of shell: C, cuticle; M, shell membranes; P, pore canal; and S, calcitic shell. (C) Changes in structure with time.

Microorganisms remain at the outer surface of the shell unless one or other of the following agents promotes their translocation along the pore canals (13): infiltration of pores by fungal hyphae; flooding of the pores with water drawn in by capillary attraction, or flooding of the pores by water when the shell is exposed to hydraulic loading. Of these the last mentioned is of major importance in the poultry industry. It is most commonly caused by immersion of warm eggs in cold water. As the egg contents contract more than the shell, a pressure difference between the contents (low) and water develops and is satisfied when water and suspended bacteria are drawn into the pore canals. Studies of this process (14, 15) and the fine structure of pores (16–18) have shown that the pores of the eggs of many species of birds are water repellent or water resistant, probably because of the nature of the nesting sites (19). With the eggs of all domesticated species of birds, water resistance is due to the cuticle, a layer of glycoprotein spheres which envelope the outer surface of the shell. Board and Halls (14) noted that many pores were easily flooded in hen eggshells that were cuticleless at oviposition or had been rendered cuticleless by chemical or physical means. Indeed there are many reports in the literature of increase levels of infection leading to rotting of eggs that were scraped or rubbed with abrasives before challenge with rot-producing bacteria (6, 13). Even with eggs having undamaged cuticles, there

are 10–20 pores (20) which for some unknown reasons provide portals for the entry of bacteria (21, 22). Such pores are often referred to as "patent pores" for no apparent reason (23).

The shell membranes consist of: an outer membrane (thickness, ca. 50–50 μm) attached to the shell at the cone layer; an inner membrane (15–17 μm) to the inside of the outer membrane except at the air cell, and a limiting membrane which separates the inner membrane from the albumen (24, 25). Electron microscopy has shown that both the inner and outer shell membranes consist of a network of fibers (Fig. 2) which lie parallel to the surface of the eggs and which are randomly oriented; the diameters of the outer membrane fibers vary from 0.4–3.6 μm whereas those of the inner membrane fibers seldom exceed 2 μm (26). Each fiber consists of a protein core separated from a glycoprotein mantle (27) by a small space (Fig. 3) which may contain moderately electron-dense material (Fig. 4). The limiting membrane is a layer of homogeneously dense material in which the innermost fibers of the inner shell membrane appear to terminate (Fig. 5).

The chemical composition of the shell membrane fibers is not fully established. Early studies classified them with keratin due to a high cysteine content (28). More recently the presence of the crosslinking amino acids, desmosine and isodesmosine, in the protein core of the fibers (29) led to the suggestion that they are elastin-like (30). Isolation of an elastin-like component has proved impossible (31, 32) and it is probable that the protein present in the membranes is unique. The cortex of each fiber has been identified as mucopolysaccharide (33) containing galactosamine, glucosamine, sialic acid, glucose, mannose, and fucose (34).

The concept that the shell membranes act as bacterial filters arose from the investigations of Haines and Moran (13). They replaced the egg contents with a suspension of bacteria and then applied suction to the shell. Fluid drawn through the shell and its membranes did not contain bacteria, but it did when the shell membranes were removed. This has been confirmed repeatedly (35, 36). When bacterial suspension and the membranes are left together for 18–24 h and then suction is applied, the filtrate is contaminated. These techniques are open to criticism because bacteria were made to traverse the shell and membranes in an opposite direction to that which they would normally travel when infecting the egg's contents. The process that results in penetration of the membranes is still unknown but it does not appear to be due to digestion of the membranes by bacterial proteases (36, 37).

In an attempt to clarify the contribution of the shell membranes, other workers have used different methods to investigate the possibility that the membranes were acting as filters. These methods included holding the shell with or without one or both of its membranes in a suspension of bacteria and recovering microorganisms from within the egg (38, 39) and inoculating the air cell and recovering microorganisms from the albumen (8, 41, 42). In summary, it appears from these studies that although the shell plus membranes offers greater resistance to penetration than does the shell alone there is little evidence to support the assumption that the outer structures of the egg are well adapted to resist bacterial penetration.

A recent observation (Fig. 6) indicates that in addition to the basic filtering technique that would appear to be the common denominator in the above studies, adhesion of bacteria to the glycoprotein mantles on the fibers of the shell membranes may well impede microbial movement also.

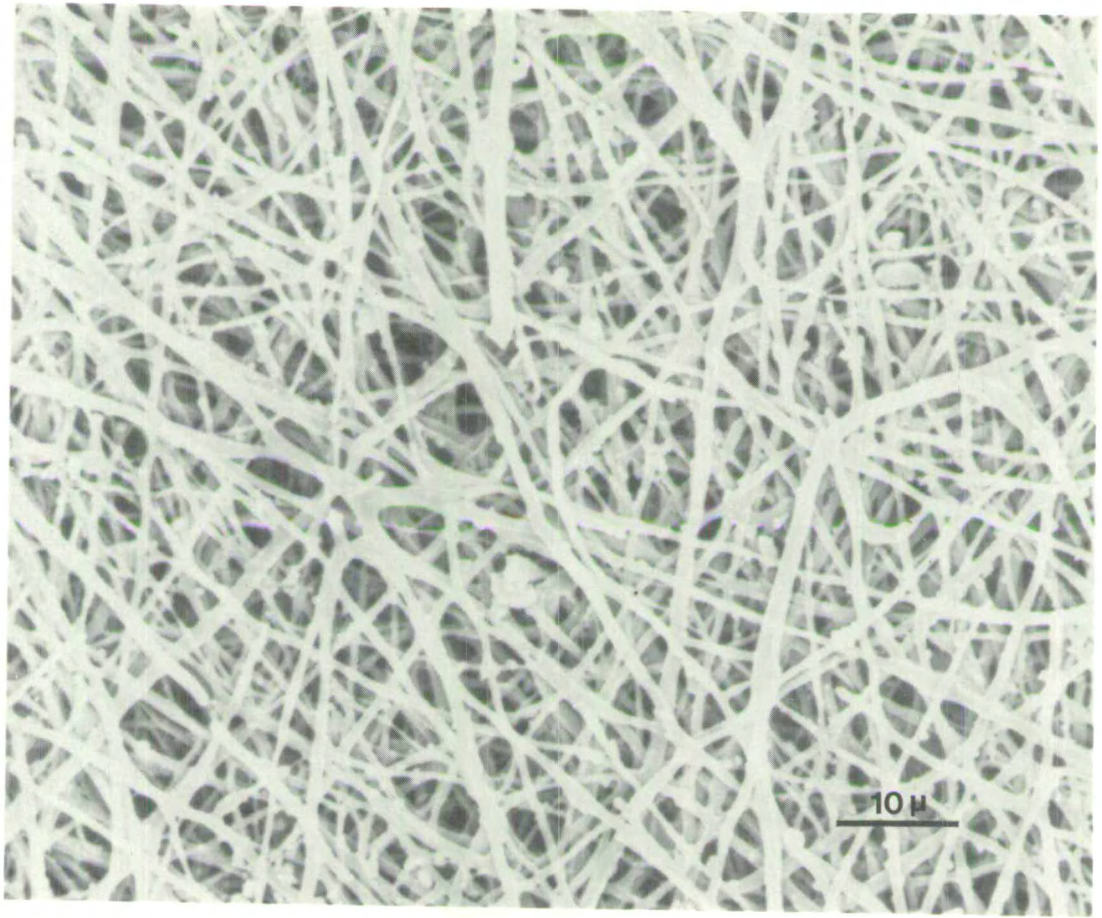

FIG. 2. Scanning electron micrograph of the shell membranes of the egg of the domestic hen (*Gallus domesticus*).

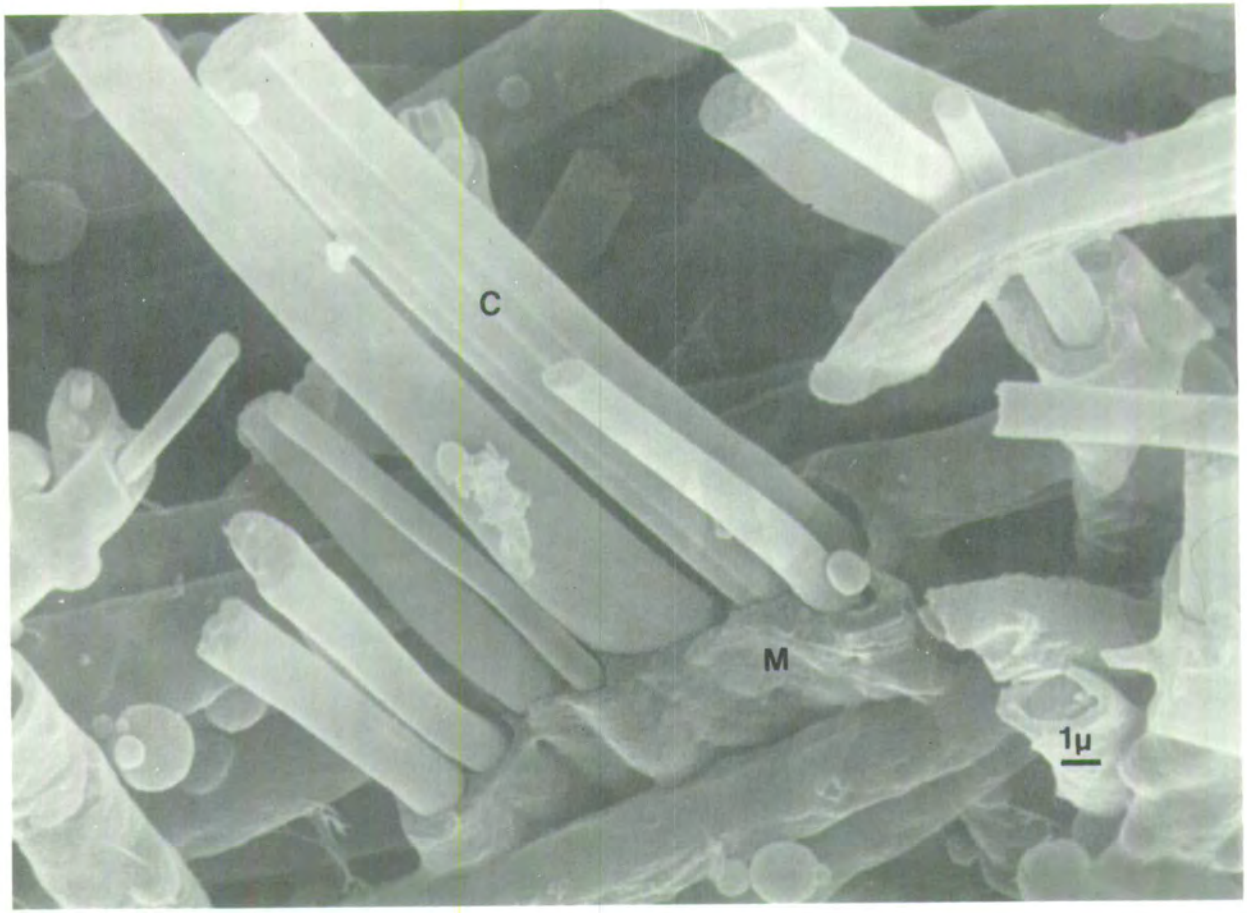

FIG. 3. Scanning electron micrograph of a fractured portion of the shell membranes of turkey eggs. Several cores (C) are contained in a common mantle (M).

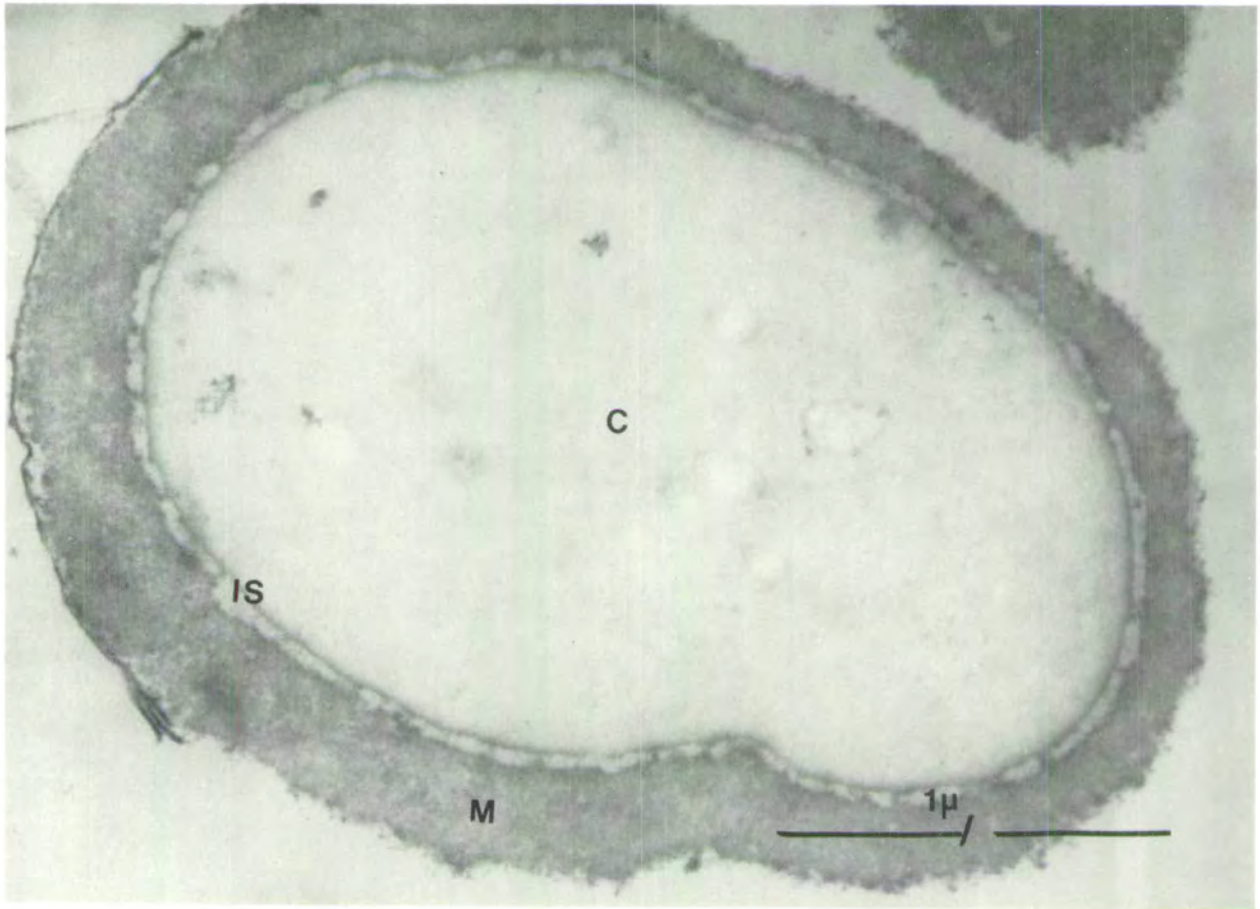

FIG. 4. Electron micrograph of a transverse section of a single fiber of the outer shell membrane of the egg of the domestic hen. C, core, and M, mantle stained with 1% (w/v) ruthenium red. The interfibrillar space (IS) is clearly visible.

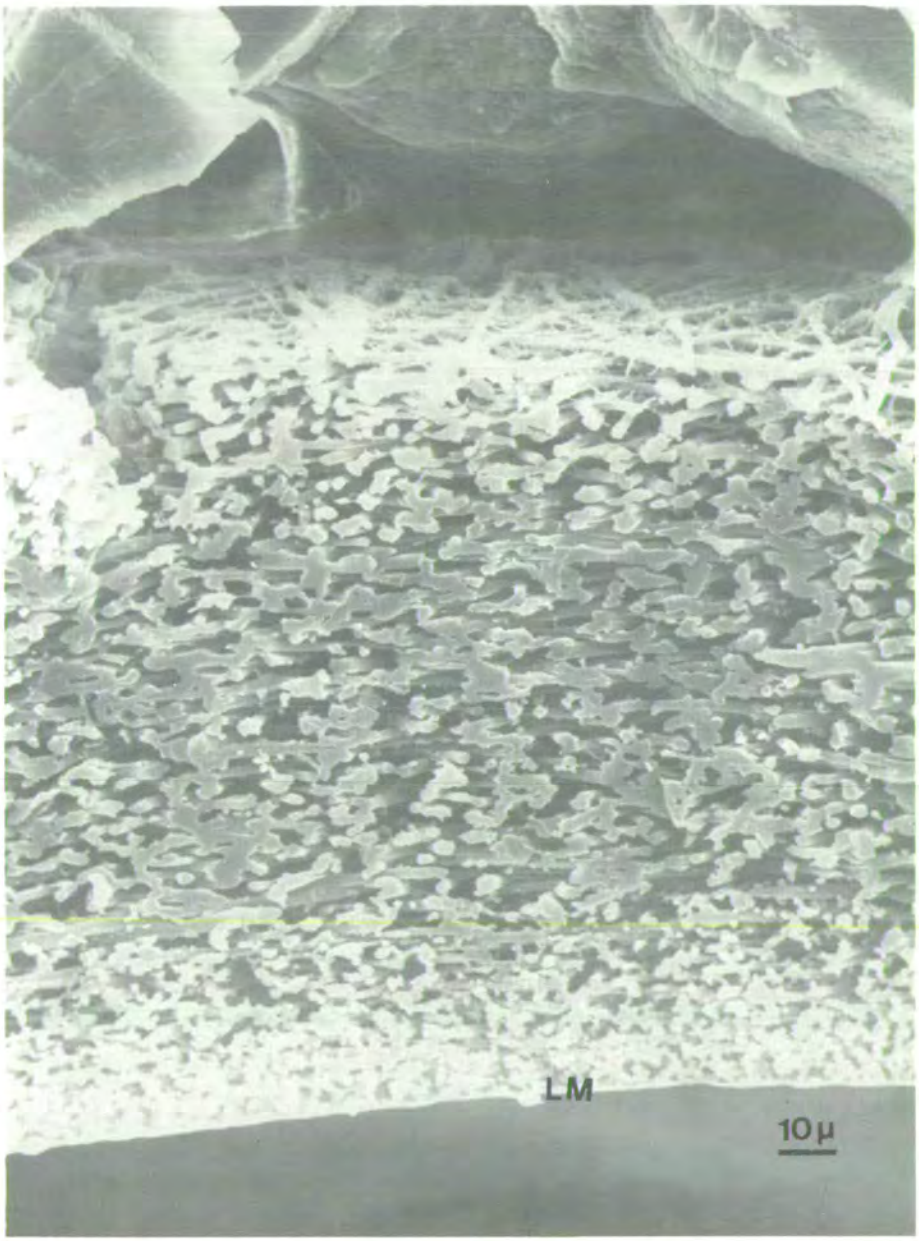

FIG. 5. The limiting membrane (LM) forms a boundary to the inner shell membrane. Freeze-fractured specimen, which was prepared by critical point drying, was examined with a scanning electron microscope (N. H. C. Sparks, unpublished observations).

Chemical Defense

It has been suggested (7) that the membranes contain antimicrobial substances that suppress microbial growth. Indeed Korotkova (43) has been quoted in the literature (e.g., 44) as having detected lysozyme in the shell membranes. A new English

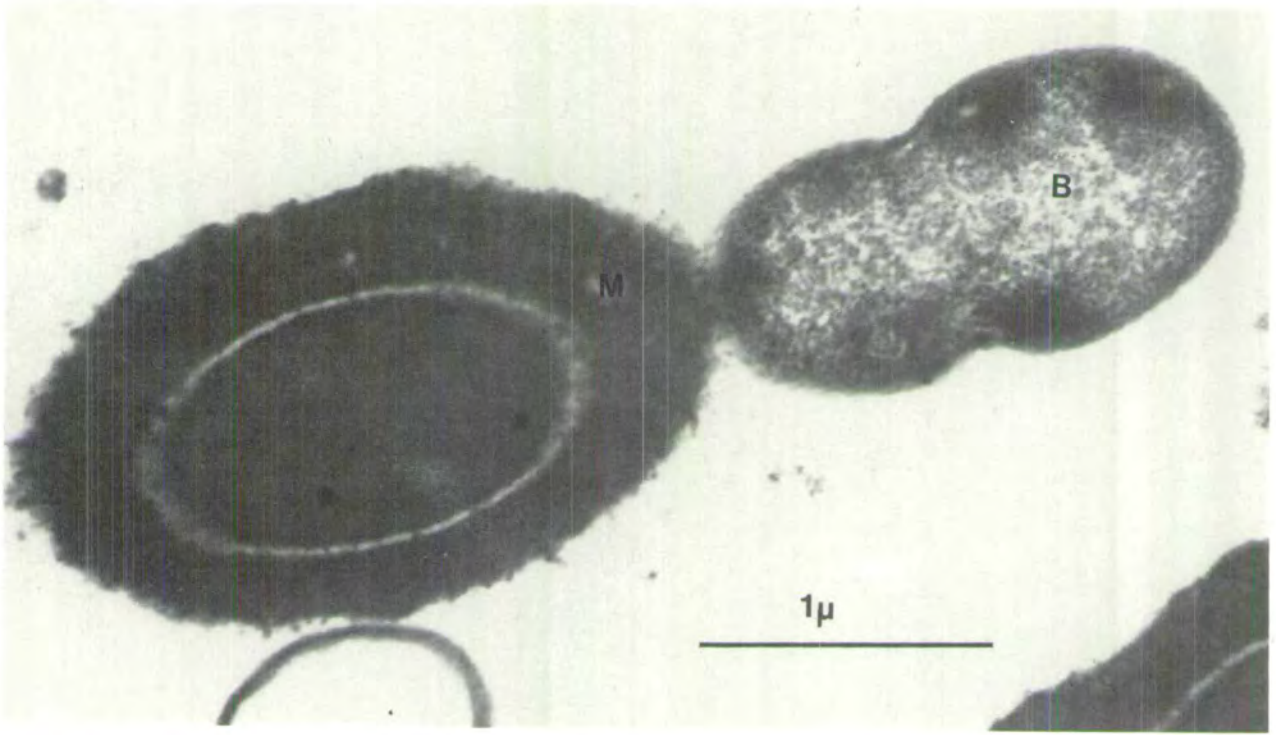

FIG. 6. A bacterium (B) adhering to the mantle (M) on the fiber in the shell membrane of the egg of a domestic hen (N. H. C. Sparks, unpublished observations).

translation from the Russian reveals that she was misquoted; she was referring to lysozyme in the underlying albumen. Rapid death of Gram-negative bacteria in a saline suspension of shell membranes has been reported (45) but not confirmed (37); microorganisms isolated from rotten eggs grow when added to shell membranes suspended in a nonbactericidal solution of mineral salts.

The colonization of the shell membranes by mixed populations of bacteria, as would occur under commercial conditions leading to infection of eggs, has received little attention (46). The limited results indicate that the early phase of infection of the membranes is characterized by the selection of nonfastidious Gram-negative bacteria. The actual rate of selection was determined by temperature which also influenced the composition of the flora, e.g., pseudomonads dominated in eggs at 4°C but coliforms in those at 37°C (42).

Many investigators have noted a lag of 15–20 days between penetration of the shells of newly laid eggs and the appearances of large numbers of organisms or macroscopic changes in the albumen (8, 42, 47–49). The early investigators attributed this to the membranes impeding microbial invasion of the albumen whereas later ones concluded that the shell membranes play only a minor role and that the antimicrobial nature of the albumen was responsible for this lag. Brooks (41) suggested that the microorganisms were restricted in growth and thereby held in the membranes because of a deficiency in iron. This view was supported by the demonstration (51, 52) that considerable bacterial multiplication occurred immediately following the seeding of the air cell with bacteria suspended in a weak solution of ferrous sulfate and that extensive contamination of the albumen occurred also.

The chemical defense occurs in the albumen, a viscous, heterogeneous material, constituting about 60% of the egg and consisting of three layers in freshly laid eggs. Each layer contains different amounts of dissolved material. The approximate percentage of total egg white:dry matter for the hen's egg is 25:21% in the outer thin white, 57:12% in the albuminous sac, and 17:13% in the inner thin white (7). The albumen differs from most other body fluids in that it consists mainly of proteins (10%) and water (80–90%). The protein content of thin and thick egg white is similar but the latter contains about four times as much ovomucin. The negligible (0.02–0.03%) amount of lipid has not been characterized. The free carbohydrate, mainly glucose, amounts of 0.5% of the albumen. There is also a wide variety of inorganic elements in dissociated and bound form. These occur in minute amounts; their concentration may be influenced by the hen's diet, environment, temperature, and age of bird (53).

Wurtz (54) was probably the first to suggest that egg white was germicidal; he noted that the typhoid bacillus and pyogenic cocci did not survive in albumen. Some subsequent workers (55, 56) repeated his observations while others (57) did not. Indeed little headway was made until the stasis or death of a test organism was associated with an identifiable component or property of the albumen. The major findings are listed chronologically below.

The first incisive study on the germicidal property of egg white was made in 1909 (55), the lysis of vegetative cells and spores of *Bacillus* spp. in egg white being observed. As this did not occur when the egg white had been heated to 65–70°C for 30 min, it was concluded that lysis was due to a thermolabile enzyme. This was confirmed by Fleming (58), who named the enzyme, lysozyme.

A rapid change in the pH (from 7.5 → 9.5) of the egg white during the week

following laying was associated (59) with the diffusion of CO_2 from the egg albumen through the pores in the shell, the rate of change being determined by temperature and the pCO_2 of the atmosphere (60). Most of the common contaminants of rotten eggs were unaffected when inoculated in albumen adjusted to pH 6–8 but were killed at pH 9–10.

The flocculation of lysis-resistant microorganisms by high dilutions of egg white was noted (61).

The growth of *Staphylococcus aureus, Escherichia coli, Shigella dysenteriae,* and *Saccharomyces cerevisiae* was inhibited in nutrient broth supplemented with egg white (62). It occurred when the mixture was adjusted to pH 7.4 or above but not to pH 5.8 or less. Of 10 vitamins and 31 elements tested, iron alone overcame the inhibition. The iron-binding substance was isolated from egg white and identified with conalbumin (63). It is now known as ovotransferrin and belongs to a class of proteins, the transferrins, which are present in various fluids of vertebrate animals (64, 65). In order to inhibit microbial growth, ovotransferrin must be present in stoichiometric excess of the iron found in a medium by chemical analysis (66, 67).

Brooks (41) was unable to obtain equivalent rates of multiplication of pseudomonads in albumen supplemented with iron and pseudomonads suspended in a favorable medium. This led him to suggest that an inadequate supply of simple nitrogenous substances might be important, as proposed earlier by Haines (6). The albumen of fresh eggs of domestic hens contains approximately 0.14–0.54 and 2.3 μmol/ml after storage for several weeks (68). These low concentrations have led to the suggestion that contaminants of the albumen would be forced to satisfy their nitrogen requirements at the expense of the egg white proteins. Such a view did not receive support (51) from studies in which the course of infection of eggs inoculated with proteolytic or nonproteolytic bacteria was compared.

During the period in which microbiologists were slowly piecing together a picture of the causes of the antimicrobial nature of the albumen, chemists were isolating many of the albumen proteins and demonstrating a range of biological properties that might be expected to contribute to the antimicrobial defense of the egg. Their achievements are reviewed in Chemical Defense Systems in the Albumen.

Biological Structure

Two explanations have been offered to account for the sudden multiplication of microorganisms in albumen following their confinement to the shell membranes (see under Integrated Working of the Chemical Defense). In one case it was attributed to a spontaneous change in the shell membranes (41, 69, 70) and in another to the chance collision of a contaminant of the albumen with the yolk (60). Recent studies suggest that two mechanisms are involved depending upon the temperature at which infected eggs are stored. Thus with storage at 10°C or less, rapid multiplication of organisms in the albumen appears to be associated with contaminants reaching the surface of the yolk (42, 52). At higher (10–37°C) temperatures, the phase of active microbial multiplication appears to be triggered off by the yolk making contact with the infected shell membranes (51). Moreover, a detailed study of the chemistry and fine structure of the shell membranes has failed to support the concept that they undergo any change, let alone a spontaneous one, during storage (71). The changes resulting in the movement of the yolk are associated with a breakdown of the al-

buminous sac (Fig. 1) and lysozyme appears to be involved (see under lysozymes and antimicrobial defense). Thus it can be concluded that the properties which characterize the albuminous sac, a weak gel interpenetrated by a system of microscopic elastic fibers (72), ensures—at least in freshly laid eggs—that the vulnerable food reserve, the yolk, is maintained at the greatest distance from potential sites of infection in the shell membranes.

The viscosity of the albumen and the properties of the albuminous sac make minor contributions to the egg's defense in that they impede motile bacteria (73).

CHEMICAL DEFENSE SYSTEMS IN THE ALBUMEN

The proteins in the albumen are unique because of their glycoprotein nature and wide range of biological properties. About 50 proteins in the albumen are easily purified because of the absence of interfering materials such as salts, sugars, or lipids. Nineteen of these proteins can be detected by starch–gel electrophoresis (74); of these, only six exist at a concentration of 1% or more. Variations in the number of electrophoretic bands of albumen from various species and inbred lines of domesticated birds have been attributed to genetic polymorphism (74, 75). The globulins (76), ovotransferrins (77–79), ovomucoid (80), and ovoinhibitor (81) all possess multiple forms. The electrophoretic patterns of the egg whites of a large number of passerine and nonpasserine birds have been used in detailed taxonomic studies (82, 83).

Many techniques have been used to fractionate and purify egg white proteins (Table I). Indeed much of the knowledge of the biological nature of albumen has come from the physicochemical properties of the proteins obtained by these methods. Until recently, these techniques had been applied to the 10 major proteins of the albumen (Table II) of the hen's egg (84) but information relating to their biological properties is perhaps not restricted to the hen because most of these proteins occur widely in birds eggs (65, 82, 83).

The physicochemical properties of those proteins considered to be involved in the antimicrobial defense system of the albumen are discussed in detail in the following sections.

Lysozyme of the Hen's Egg

Fleming (58) is considered to have been the first person to associate bacterial lysis in egg white with the action of an enzyme for which he coined the name, lysozyme (a muramidase—*N*-acetylhexosaminidase, EC 3.2.1.17). Although various lysozymes have been isolated subsequently from the albumen of eggs of various species of birds, the following discussion will be concerned with the lysozyme$_c$, of the egg albumin of domestic hens (*Gallus domesticus*).

It was shown in early studies (85) with lysozyme that its substrate occurred in the cell of "*Micrococcus lysodeikticus*," an organism isolated and used by Fleming because of the ease with which it could be lysed. Confirmation of the site of the substrate came from studies in which osmotically fragile protoplasts of *Bacillus megaterium* (86) and sphaeroplasts (87) of *Escherichia coli* were isolated from cultures to which lysozyme had been added. These observations showed therefore that lysozyme was not a bactericidal agent per se, it merely initiated events leading to death by breaching the rigid eubacterial cell wall and thereby exposing the underlying cell membrane to rupture if the suspending fluid was hypotonic.

TABLE I

Common Methods for the Purification of Avian Egg White Proteins

	Reference
Lysozyme	
Direct crystallization from egg white containing 5% (w/v) NaCl at pH 9.5	295
Ion-exchange chromatography on carboxymethyl cellulose (CMC)	217
Ion-exchange chromatography on diethylaminoethanol cellulose (DEAE)	296
Ion-exchange chromatography followed by gel filtration	111
	297
Ovotransferrin	
$(NH_4)_2SO_4$ fractionation from egg white	298
Ethanol fractionation from egg white	299
Ion-exchange chromatography on carboxymethyl cellulose	217
Crystallization from egg white using $(NH_4)_2SO_4$ followed by ion-exchange chromatography	300
Avidin	
Acetone and salt fractionation from egg white	182
Absorption onto bentonite	205
Ion-exchange chromatography on CMC	217
	301
Affinity chromatography using Sepharose	302
CMC chromatography followed by crystallization	192
Ovoflavoprotein	
DEAE chromatography followed by chromatography	219
	224
Ovomucoid	
Trichloroacetic acid (TCA) precipitation of egg white proteins at pH 3.5 followed by acetone precipitation of the TCA-soluble fraction	234
Ion exchange chromatography	236
Ovoinhibitor	
Ion-exchange chromatography of TCA precipitated ovomucoid	253
	256
$(NH_4)_2SO_4$ fractionation followed by gel filtration and DEAE chromatography	254
Ficin and papain inhibitor	
$(NH_4)_2SO_4$ fractionation followed by gel filtration and ion-exchange chromatography	257

The cell wall of "*M. lysodeikticus*" is formed in part from a highly water-insoluble polymer, peptidoglycan, which consists of linear strands of alternating units (Fig. 7) of *N*-acetylglucosamine (NAG)[2] and *N*-acetylmuramic acid (NAM), β (1–4) linked and interconnected by peptide chains (Fig. 8) attached to the D-lactyl group of some (50%) of the NAM units. The β (1–4) linkages (Fig. 7) between NAM and NAG residues are attached by lysozyme (88, 89). It attacks also the β (1–4)-NAG-linked units of chitin (88). Thus certain conditions have to be fulfilled before an organism is lysed by lysozyme. In the first instance, its cell wall must contain an appropriate peptidoglycan. Lysozyme resistance in *Bacillus cereus*, for example, has been attrib-

[2] Abbreviations used: NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; CNBr, cyanogen bromide; TPCK, 1-tosylamide-2-phenylethylchloromethyl ketone.

TABLE II

Some Properties of the Main Proteins of Hen Albumen

Protein	*Amount in albumin (%)*	M_r	*pI*	*Characteristics*
1. Ovalbumin	54	46,000	4.5	Phosphoglycoprotein
2. Ovotransferrin	12	80,000	6.05	Chelation of metal ions particularly iron
3. Ovomucoid	11	28,000	4.1	Inhibition of trypsin
4. Lysozyme	3.4	14,600	10.7	Hydrolysis of β (1–4) glycosidic bond in peptidoglycans Electrostatic interaction with ovomucin
5. Ovomucin	3.5	ND[a]	4.5–4.7	See No. 4, lysozyme
6. Ovoinhibitor	1.4	44,000–49,000	5.1	Inhibition of several proteases
7. Ovomacroglobulin	0.5	760,000–900,000	4.5	—
8. Ovoglycoprotein	1.0	24,400	3.9	—
9. Ovoflavoprotein	0.8	32,000	4.0	Chelation of riboflavin
10. Avidin	0.05	70,000	9.5	Chelation of biotin

[a] Value not determined or reported.

uted to the absence of *N*-acetyl groups on the glucosamine (90). Sensitivity to a limited extent can be induced by *N* substitution with acetyl, propionyl, butyryl or formyl groups (91). With Gram-positive bacteria, for example *Staphylococcus aureus*, resistance to lysozyme can be caused by cell wall accessory materials. Thus teichoic acid, a highly negatively charged component of the cell walls of the cited organism and many other Gram-positive bacteria, binds a basic protein such as lysozyme thereby preventing the latters diffusion to its substrate. Peptidoglycan makes a relatively small contribution (5–10%) to the cell walls of Gram-negative bacteria (92) and it is buried beneath laminae of lipoproteins and lipopolysaccharides (Fig. 9), which impedes the diffusion of lysozyme. These barriers may be perturbed experimentally by incubation under alkaline conditions (87, 93), freezing and thawing (94), or treatment with alkaline (pH 8–9) ethylenediaminetetraacetic acid (95).

Lysozyme$_c$, a basic protein with a M_r of about 14,000, consists of a single polypeptide chain of 129 amino acids (96, 97) crosslinked in four places by disulfide

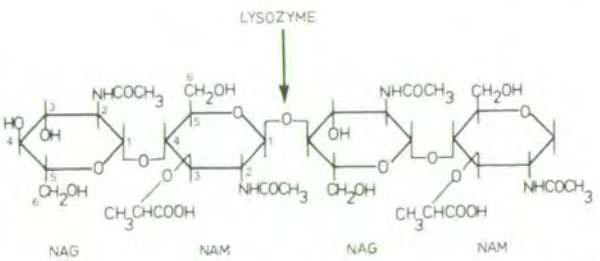

FIG. 7. Schematic diagram of a eubacterial cell wall tetrasaccharide showing the β (1–4) linkage that is attacked by lysozyme$_c$.

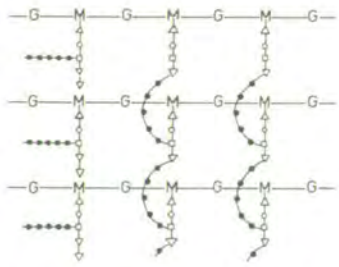

FIG. 8. Schematic diagram of the basic structure of bacterial cell wall peptidoglycan. G = *N*-acetylglucosamine; M = *N*-acetylmuramic acid; △ = L-alanine; ○ = D-isoglutamine; □ = L-lysine; ▽ = D-alanine; ● = glycine. The pentaglycine bridges on the left are in amide linkage to lysine but have not yet been connected to the carboxyl of D-alanine. The completed pentaglycine bridges are all shown connecting parallel polysaccharide chains in the plane of the paper. However, they can extend to equivalent peptidoglycan sheets above or below the plane of the paper. Adapted from (40).

bridges (98). The three-dimensional structure of the molecule has been determined by X-ray analysis (99–102).

The sequences of amino acid residues 5–15; 24–34; 88–96 form three lengths of α helix although these appear to be distorted from the "classical" form. The helical sections tend to be rotated so that their –CO– groups point outward and their –NH– groups inward from the axis of the helix. This rotation is necessary for the internal hydrogen bonding of the molecule. There are two lengths of anti-parallel pleated sheets which occur between residues 41–45 and 50–54. Residues 46–49 are folded into a hair-pin bend between the two lengths described above. The whole structure is stabilized by hydrogen bonds but the remainder of the chain, other than the residues described, is folded in irregular ways that defy simple description. Phillips (101) commented on the folding of the lysozyme molecule. The folded chain forms a structure with two wings lying at an angle to each other (Fig. 10). The length of α

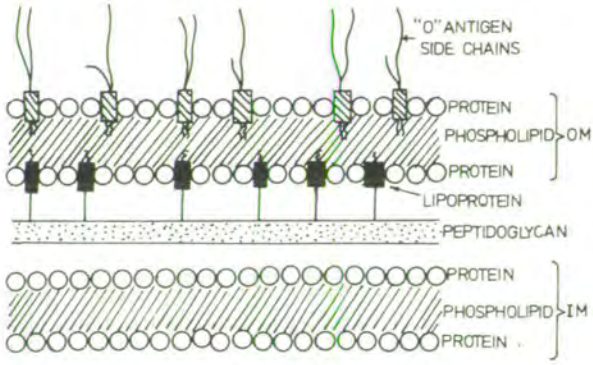

FIG. 9. Schematic representation of a Gram-negative bacterial triple layered cell wall. The trilaminar cytoplasmic or inner membrane (IM) is covered by a thin (20–30 Å) layer of peptidoglycan and a trilaminar outer membrane (OM), which is attached to the peptidoglycan by a continuous layer of lipoprotein embedded in the former and covalently attached to the latter. The outer membrane also contains lipopolysaccharide which is accessible to antibodies. Adapted from (50).

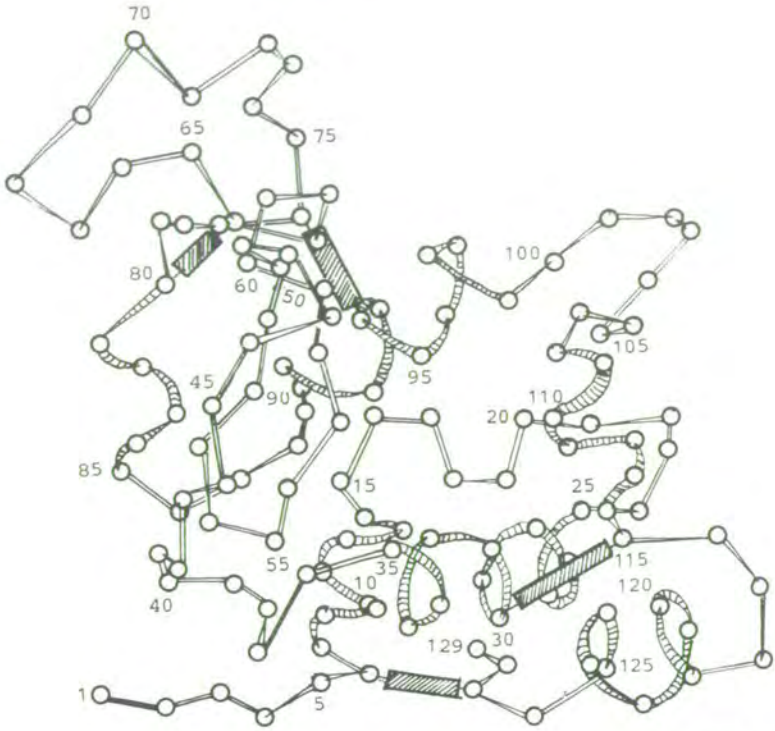

FIG. 10. Schematic representation of the tertiary structure of lysozyme$_c$ of hen egg white. Based on Ref. (101).

helix formed by residues 86–96 lies in a gap formed between the two wings but does not completely fill it. The remaining cleft forms the active site.

Lysozyme is inhibited (99) by the chitin trisaccharide, $(NAG)_3$. It binds to the enzyme molecule by six hydrogen bonds and over 40 Van der Waal's contacts, filling nearly half the length of the cleft. Inhibitors longer than three sugar residues cannot be used because they are more readily cleaved and have difficulty diffusing into the enzyme crystal. Based on the assumption that the interactions involved in the binding of the inhibitor were the same as those involved in the binding of the substrate, a model has been constructed (101) which shows how a substrate consisting of six residues (A–F) of NAG could bind to the cleft. It showed that: (a) six sugar residues fill the entire length of the cleft; (b) residue D is distorted from its normal chair conformation to a half-chair conformation; (c) *N*-acetylmuramic acid could not bind to the enzyme at subsites A, C, or E due to the D-lactyl ether group at C3; (d) the only possible linkage affected by the enzyme was between sugar residues D and E; (e) the most reactive groups near subsites D and E are the carboxyl groups of GLU 35 and ASP 52 which are located on either side of the β (1–4) linkage. A movement of the side chain of TRP 60, about 0.75 Å toward sugar residue B, was observed (99) when the substrate was bound to the enzyme thus indicating an induced fit (103).

In experiments with ^{18}O-labeled substrates, it was shown (104) that lysozyme-catalyzed reactions involved cleavage between C-1 of one pyranose ring and the oxygen joining it to C-4 of the next ring. Glycosides are especially susceptible to acid-

catalyzed carbonium ion formation because the neighboring ring oxygen stabilizes the positive charge. In glycopyranosides, however, a conformational change is necessary for this to occur. The enzymatic mechanism has been postulated to take place in the following manner. A lysozyme molecule attaches itself to the bacterial cell wall by interacting with six exposed amino sugars providing other components—e.g., teichoic acids—do not prevent the enzyme from attaching. During this process, residue D is distorted from its usual conformation. GLU 35 then transfers its terminal hydrogen atom in the form of a hydrogen ion to the glycosidic oxygen bringing about the cleavage of the bond between that oxygen and C-1 of residue D. This creates a positively charged carbonium ion (C^+) where the oxygen has been removed from C-1. This ion is stabilized by interaction with the negatively charged side chain of ASP 52 until it can combine with a hydroxyl ion (OH^-) that diffuses into position from the surrounding water. The lysozyme molecule then falls away leaving behind a punctured cell wall and a cell that may lyse in hypotonic media. The reactive groups near the bond cleaved are in environments that favor this catalytic action, i.e., ASP 52 is in a highly polar environment and is probably ionic under most conditions—GLU 35 is in a nonpolar environment and is unlikely to be ionised. Table III lists a few of the many interactions involved in the binding of the substrate to the enzyme.

A "transglycosylation" action for lysozyme has been reported (105, 106). This proceeds in the same way as hydrolysis but, instead of water completing the reaction, an acceptor saccharide attaches to the cleft at subsites E and F and is bound to the residues left behind in the cleft.

In laboratory studies the decrease in turbidity with time of a suspension of lyophilized cells of "*M. lysodeikticus*" is the commonest method of demonstrating lysozyme activity (107). Lysis occurs over the pH range of 4–10 (81, 108, 109). The rate of lysis is markedly dependent upon ionic strength; lysozyme is activated by low and inhibited by high salt concentrations. This inhibition is correlated closely with the cation concentration and charge (108) so that polyvalent cations are stronger inhibitors than monovalent ones. It has been suggested (81) that inhibition is due to the high ionic strength disrupting the electrostatic forces that attach the lysozyme molecule to the bacterial cell wall.

Other Lysozymes

Lysozymes which differ in certain respects—for example amino acid sequences (110, 111)—from lysozyme$_c$ of the albumen of domestic hens have been isolated

TABLE III

Some of the More Important Interactions between Lysozyme and Its Substrate

Amino acid residue	*Type of bond*	*Interaction*
ASP 101	H Bond	A Ring amide NH
ASP 101	H Bond	B Ring C6
TRP 62	H Bond	C Ring C_6OH
TRP 62	v.d. Waal	B Ring
TRP 63	H Bond	C Ring C_3OH
ASN 59	Main chain H Bond	C Ring amide C=O
ALA 107	Main chain H Bond	C Ring amide N–H

from the egg albumen of other species of birds (65). Moreover, there is a pronounced variation in the concentration of these enzymes in eggs, viz., 3–4% (wet wt) in the albumen of the eggs of chicken, other galliformes, and anseriformes (65) to only trace amounts in that of penguin eggs (112).

Initial studies of the lysozyme of White Embden goose egg white led to the interesting observation that it differed significantly from that of chicken egg white and other homologous avian lysozymes in amino acid sequence (113), enzymatic activity (114–116), crystallography (117), and immunogenicity (110, 118). The goose lysozyme, lysozyme$_g$, has approximately 180 amino acids, 4 half-cysteine residues, 2 disulfide bridges, and a M_r of 19,500–21,500. It is a muramidase like the lysozyme from chicken, lysozyme$_c$ (115), but it is specific for NAM residues whose lactyl group is peptide linked (119). In contrast to lysozyme$_c$, it has a marked inability to hydrolyze large polymers of NAG (120) and is not affected by NAG inhibitors (114).

The lysozyme molecule contains amino acid residues arranged in "sites" to which antibodies may bind (121–123). Antisera produced in response to lysozyme$_c$ will not cross-react with lysozyme$_g$ and vice versa. Thus serological methods can be used to demonstrate the presence of these two in other egg whites, for example both lysozyme$_c$ and lysozyme$_g$ occur (124) in the egg white of the Black Swan. Available evidence suggests that these are the products of different genetic loci. The egg white of other birds contains only lysozyme$_c$ (chicken, Peking duck) or lysozyme$_g$ (Embden goose). The absence of either enzyme is not a reflection on whether or not the structural genes for these lysozymes are present in the genome of the species; both genes are present and for some reason only one is expressed in some instances. A tissue survey of the duck revealed both types of enzyme (125). The domestic hen produces both lysozyme$_c$ and lysozyme$_g$ also, the former occurring in the egg white and both in the polymorphonuclear leukocytes (126).

Lysozymes and Antimicrobial Defense

A discussion of this topic must be restricted to lysozyme$_c$ because little work has been done on the others. Although the lytic action of lysozyme in albumen has been demonstrated with lysozyme-sensitive bacteria (58, 127) there is no direct or compelling evidence that it plays an important role in protecting avian eggs against infection (128). Russian workers (43, 129, 130), who have taken a broad biological view of the role of the defense systems of avian egg, have concluded also that this enzyme plays but a minor role. Moreover we have been unable to induce lysozyme sensitivity by culturing *Escherichia coli* in an alkaline (pH 9.0) medium in which iron was chelated with ovotransferrin (Fig. 11). This result suggests that the interaction of several or all of the antimicrobial components of the albumen is unlikely to damage the cell walls of Gram-negative bacteria to such an extent that lysozyme can diffuse to its substrate.

Lysozyme may be involved more directly in the physical structure of the white and hence the physical defense of the egg (see under Biological Structure). The gelatinous structure of the thick white is formed by an interaction between lysozyme and ovomucin, the structural glycoprotein of egg white (72, 131–133, 135). Hen egg white ovomucin consists of two components (134–137): ovomucin (M_r 210,000) containing about 1% (w/w) sialic acid and β ovomucin (M_r 720,000) which consists of globular subunits and contains 10% (w/w) sialic acid. It has been shown (137) that

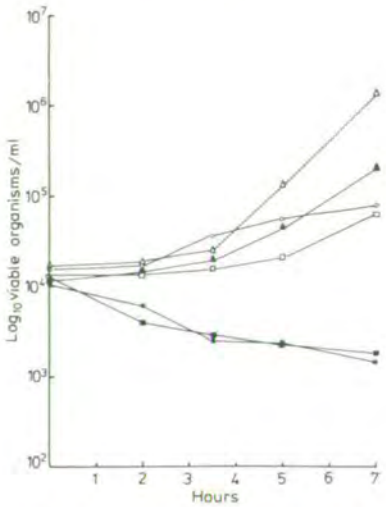

FIG. 11. The effect of lysozyme$_c$ (4 mg/ml) on *Escherichia coli* 0111 inoculated in M9 minimal medium pH 7.0 (open symbols) or pH 9.0 (solid symbols) in the presence of unsaturated (○, ●) or Fe^{3+}-saturated ovotransferrin (△, ▲). Ovotransferrin concentration, 1.26 mg/ml (H. S. Tranter, unpublished observations).

there is an electrostatic attraction between the negative charges of the terminal sialic acid and the positive charges of the lysyl ϵ-amino groups of lysozyme and that this interaction decreases correspondingly to enzymatic removal of the sialic acid by neuraminidase. As the internal quality of the egg has been related to the lysozyme content of thick egg white (138, 139), it would seem reasonable to assume that lysozyme may contribute in two ways to the antimicrobial systems of the egg white of domestic hens, viz.: (a) lysis of sensitive microorganisms and, more importantly, (b) maintenance of the albuminous sac and hence the greatest distance between the probable nidus of infection, the shell membranes in eggs stored at or above ca. 10°C, and the vulnerable food reserves in the yolk.

Ovotransferrin

The transferrins, a family of homologous proteins, are distributed widely in the sera of most vertebrates (140) in the milk and other secretions of mammals (141) and in the white of avian eggs (82, 142, 143). No particular nomenclature has been generally accepted for these proteins and many names are used in the literature:

Iron binding protein from serum:	transferrin; siderophilin; β_1-metal combining protein.
Iron binding protein from milk:	lactotransferrin; lactoferrin; red protein.
Iron binding protein from egg white:	conalbumin; ovotransferrin.

A characteristic feature of all of these proteins is their ability to bind two ferric iron atoms per protein molecule (144) at two different sites (145) to produce a salmon-pink complex (146) with an absorption maximum at 400–470 nm; this forms the basis of many assay systems for these proteins.

The transferrins may form complexes with other transition metals; these have absorption maxima at different wavelengths and absorbances of different magnitude. Thus, copper yields a yellow complex with transferrin (147) and ovotransferrin (66) which has an absorption maximum at 440 nm, but the zinc–ovotransferrin complex is colorless (148). Displacement studies (149) showed the following relative stabilities of metal–ovotransferrin complexes:

$$Fe^{3+} > Cr^{3+}; Cu^{2+} > Mn^{2+}; Co^{2+}; Cd^{2+} > Zn^{2+} > Ni^{2+}.$$

The metal complexes dissociate in acid (<pH 6.5) but not in alkaline solution (pH 9–10). Divalent iron is not bound to transferrin (150) although claims have been made (151) that transferrin possesses ferroxidase activity and that Fe^{2+} is bound to transferrin before being oxidised by atmospheric oxygen.

Starch gel electrophoresis has revealed that ovotransferrin exists in multiple forms. This is particularly notable in the starch–gel patterns of the Ratites (152). The cassowary, for example, possesses six forms of ovotransferrin (143) which can be separated one from another. Chicken egg white ovotransferrin can vary electrophoretically and genetically within one commercial strain (74, 77). Methods for the purification of ovotransferrin are given in Table I.

Ovotransferrin is a glycoprotein constituting about 13% of the total protein content of hen egg white. Its carbohydrate moiety consists of 3.5 mol mannose and 5.6 mol of glucosamine per 80,000 g of protein (153); most (88%) of this carbohydrate occurs as a single oligosaccharide. Ovotransferrin, unlike transferrin, contains no sialic acid (154).

Conflicting observations have been made by workers who have attempted to define the primary structure of ovotransferrin. The amino acid composition is not unusual and does not vary appreciably between ovotransferrins from different avian species (152). Suggestions that its high molecular weight (88,000–95,000) was evidence of major subunits have not been supported by chemical (155) or physical techniques (156, 157). Indeed it appears to consist of a single polypeptide chain. The suggestion (156) that the chain contains two homologous sections, each of which originates by gene duplication, was supported by the observations (158) that ovotransferrin is split into three polypeptide fragments by cyanogen bromide cleavage, whereas nine fragments would have been expected from the methionine content of the protein. The sum of the molecular weight of these fragments (21,000:9400:7000) was exactly half that of the native protein. Other workers have obtained results that suggest ovotransferrin does not consist of identical halves, the molecule being split into eight (159) or four, and following carboxymethylation, eight pieces (160–162) one of which (fragment CF1) had specific iron binding activity. The other binding site was associated with the remaining three fragments, CF2, CF3, and CF4.

Unlike hemoglobin, ovotransferrin does not have prosthetic groups involved in the binding of Fe^{3+}. It was postulated (66) initially that the metal-binding site in ovotransferrin was associated with the side groups of specific amino acid residues and that these were similar for each of the two sites. As the binding of iron and copper to ovotransferrin was shown to be accompanied by the release of three and two protons, respectively, it was postulated (148, 163) that, with the pH at which they worked, tyrosyl groups would be the most likely source of these protons. This was confirmed by the demonstration that no color developed when iron or copper was added to ovotransferrin which had its tyrosine groups iodinated (164); there was

no loss of color when the metal complex was iodinated. Acetylation (165) and nitration (166) of ovotransferrin confirmed the contention that tyrosines were involved in the metal-binding site. Indeed with the last two methods, six fewer tyrosyls were modified in the iron-saturated than in the iron-free protein thereby suggesting that three tyrosyls were involved in the binding of each iron atom. Modification of the imidazole groups in transferrin with bromoacetate produced (166, 167) an apotransferrin (metal-free transferrin) which could not bind iron. Two imidazole groups were found to be bonded to each iron atom.

Schade *et al.* (146) were the first to demonstrate that CO_2 was involved in the formation of iron complexes of human serum transferrin and Warner and Weber (148) that ovotransferrin required carbonate or bicarbonate for the formation of the colored complex. Iron binding to the specific sites on the transferrins needs concomitant anion binding (168); carbonate or bicarbonate is the preferred anion but small molecular weight compounds possessing two or more carboxyl groups (oxalate, EDTA) will also activate the metal binding site (168, 169). The anion is necessary for chromophore development (168) and blocking the carboxyl groups with glycinamide prevents or glycine ethyl ester slows down color formation (169). It has been shown also (168) that ternary complex formation between metal, chelate, and transferrin takes place in the absence of bicarbonate thus suggesting that bicarbonate, through the displacement of the chelate, was involved in the rate-limiting step of chromophore development. This view was supported by the kinetic data obtained from the binding of iron and copper to hen ovotransferrin (170). Other workers (e.g., 171) have surmised that transferrin appears to have no affinity for iron in the absence of anions, the anions serving as binding ligands between the metal and protein thereby stabilizing the bond and protecting it from hydrolysis (172). Although a model (Fig. 12) of the iron-binding site involving the anion has been proposed (173), it does not account for other ligands such as the hydroxyls of tyrosine and the imidazole nitrogen of histidine involved in the binding site.

The two iron-binding sites of ovotransferrin were considered to be equivalent and independent of each other (144). Measurement of the binding by equilibrium dialysis (148) showed that the binding constants for iron are very large (ca. 10^{30}) and that the two stoichiometric binding constants do not differ appreciably (174). It was proposed (175) and confirmed (176) that ovotransferrin exists in three forms at equilibrium: (a) iron free, (b) complexed with one Fe^{3+}, and (c) complexed with two Fe^{3+}. Moreover in the absence of low-molecular-weight chelators such as nitrilotriacetic acid (NTA), iron binds randomly to the two sites; in the presence of NTA, binding at one site is slightly favored and once iron has been bound at either site the binding affinity for the unoccupied one is decreased (176). This "anti-cooperativity" may be

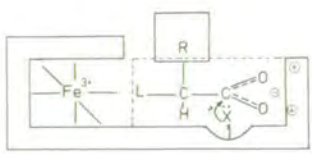

FIG. 12. A hypothetical model for the binding of Fe(III) and carbonate to transferrin: L = proximal ligand coordinated to Fe^{3+}; R = large component that projects from the surface of the molecule; X = protein component possessing a positive charge which makes the carboxy carbon atom susceptible to nucleophilic attack. Taken from (171).

attributed (177) to a conformational change in the protein following the binding of the first Fe^{3+}. Unlike human serum transferrin where marked differences in the release of the first and second Fe^{3+} ions occur (178, 179), the release of this element by ovotransferrin to chick embryo erythrocytes does not distinguish between the two sites (172).

The iron complexes of ovotransferrin are more stable than the metal-free ones to denaturation by heat, organic solvents, high pressures, and exposure to high concentrations of urea or guanidine (164, 180). This enhanced stability has been interpreted on the basis that chelation of the metal causes the formation of new bonds that stabilize and perhaps modify the secondary and tertiary structures of the molecule. The formation of the iron complex stabilizes the active center for iron binding so that the capacity is protected but also that modification of other parts of the molecule can occur without denaturation.

The biological role, if any, of ovotransferrin in embryogenesis has not been elucidated. There is little doubt, however, that it is the principal component of the antimicrobial defence of eggs (see under Integrated Working of the Chemical Defense); through depriving microorganisms of iron it causes inhibition of their growth (Table IV).

Avidin

This protein of hen egg white (181, 182) forms a nondigestible complex with biotin (183) such that the vitamin is made unavailable to microorganisms that require it. It is present in the egg white of many birds (184, 185) as well as the albumen-secreting tissues of the oviduct of laying but not nonlaying hens (186). Its synthesis can be induced also by adding progesterone to the oviduct of estrogen-primed chicks (186–188). Biotin-binding proteins have also been found in the egg yolk (189) and plasma (190) of the hen. These are normally saturated with biotin which exchanges quite readily at body temperature, an important feature if biotin is to be available for the developing embryo. In contrast, avidin occurs in an unsaturated state in the egg white; it binds biotin so tightly that little exchange takes place at body temperature.

Avidin (Table II), a basic glycoprotein (M_r 70,000), consists of four identical poly-

TABLE IV

The Effect of Iron Saturation of Ovotransferrin on the Fate of *Escherichia coli* C3650 in Egg White at 39.5°C

Percentage saturation of ovotransferrin with iron[a]	*Viable count cells/ml albumen*			
	0 h	*6 h*	*12 h*	*24 h*
0	7.73×10^3	9.83×10^2	1.0×10^2	—
25	7.70×10^3	1.04×10^3	2.5×10^2	—
50	8.1×10^3	1.72×10^3	2.75×10^2	—
75	7.79×10^3	1.48×10^3	5.0×10^2	1.0×10^2
100	7.95×10^3	1.22×10^4	1.5×10^6	7.2×10^8

Note. From H. S. Tranter, unpublished observations.

[a] The different saturation values were calculated from the amount of iron needed to give 100% saturation of hen ovotransferrin, as determined by a standard curve.

peptide subunits containing 129 amino acids (191, 192), with alanine and glutamate at the amino and carboxyl ends, respectively, and an uncharacterized carbohydrate moiety. Trypsin and cyanogen bromide (CNBr) cleavage have established the complete amino acid sequence (191, 193, 194). The protein is characterized by its high threonine content, single residues of histidine and tyrosine, and two of cystein. It also contains four to five residues of mannose and three to four of glucosamine linked by one of the acetylglucosamine residues to ASN 17.

Avidin binds four molecules of biotin, one per subunit. Measurement of the dissociation constant by equilibrium dialysis with radioactive biotin (195) gave an upper limit of 10^{-10} M. An accurate determination was impossible because of impurities in the biotin. The rate of exchange of bound [^{14}C]biotin with an excess of unlabeled biotin by separating free from bound biotin with CM-cellulose gave a value of 10^{-15} M (196).

The binding site of avidin does not contain reactive amino, phenolic, imidazole, carboxyl, or disulfide groups and significant (>70%) inactivation results from oxidation with H_2O_2 in the presence of Fe^{2+}, treatment with formaldehyde in the presence of alanine or NH_2OH at 50°C (197). Although these early observations did not permit precise interpretation, they did implicate tryptophan residues. Indeed subsequent studies of the oxidation of tryptophan by *N*-bromosuccinimide indicated that each molecule of biotin protects four tryptophan residues from oxidation, and that each subunit acts independently of the others (196, 198, 199). Tyrosine and tryptophan account for 96% of the absorbance of avidin at 282 nm. The shift of this spectrum to longer wavelengths when avidin reacts with biotin also suggests that tryptophan residues are involved in the binding (200). The shift has been interpreted also as a transfer of tryptophan into a less polar environment on binding to avidin. A lysine residue has also been implicated in the binding of biotin to avidin (201).

Biotin analogs also produce changes in the difference spectra on binding to avidin and their dissociation constants can be calculated from the spectrophotometric titration curves (196), the dissociation constant decreasing as the similarity of the analog to biotin becomes more remote. This provides clues about the different parts of the biotin molecule required for binding. For example, analogs containing a broken imidazolidone ring have dissociation constants 10^7 times greater than that of biotin. Measurement of the free energy associated with the binding of biotin and its analog to avidin indicate that every atom in the biotin molecule is involved in the interaction with avidin.

Avidin, and particularly the avidin–biotin complex, has a marked stability to denaturation by heat or breakdown by proteolytic enzymes (202). The ionic strength of the heating medium is important; the release of biotin from avidin by autoclaving (203, 204) was more rapid (88% complete after 10 min at 100°C) in the absence of 0.2 M ammonium carbonate than it was in its presence (10% after 15 min at 100°C).

Avidin resists unfolding in high concentrations of urea (8 M) (205) and moderate concentrations of guanine hydrochloride (206). Above 3.5 M GuHCl, the protein begins to unfold and its binding ability decreases concomitantly. Molecular weight measurements at 6 M GuHCl indicate that the protein dissociates into monomers. Tertiary structure and binding ability are not regained until the concentration of GuHCl is lowered to 2 M. Although at 3.5 M GuHCl there is no detectable tetramer structure, biotin is bound to refolded monomers. Saturation of avidin with biotin prevents unfolding even in 8 M GuHCl or 0.1 M HCl. In partially saturated avidin

only the unoccupied subunits are vulnerable to denaturation by GuHCl (192) or oxidation by *N*-bromosuccinimide (199). The denatured subunits dissociated into monomers and the protected ones recombine to form tetrameric avidin–biotin complexes. This indicates that the binding site is almost certainly situated within a subunit rather than at an interface between subunits. It has been shown (192) moreover that biotin could be bound to monomers coupled to a Sepharose matrix to prevent them from reassociating. Formation of active subunits was not dependent on interactions with other subunits but interaction was required for firm binding of biotin.

Studies (207) of fluorescence quenching have shown that binding to avidin is a random process and that there is no detectable interaction between the sites. Optical rotation and optical rotatory dispersion studies (200, 208) indicate that binding does not result in any gross morphological changes of avidin. This is supported by the observations (209) that crystals of avidin and the avidin–biotin complex are isomorphous. Crosslinking of avidin molecules with bisbiotinyldiamines (210) has been used to determine the spatial relationships of the binding sites in the subunits. The binding sites are grouped in two pairs at opposite ends with a dimension of 55 × 55 × 41 Å.

The early methods of estimation of avidin were based on its ability to deprive biotin-requiring microorganisms such as yeasts (211) or *Lactobacillus* spp. (212, 213) of biotin. Although extremely sensitive these methods were time consuming. As there is an appreciable spectral change (a new absorption band appears $\epsilon_{500} = 34{,}000$) when the dye 4-hydroxybenzene-2-carboxylate is bound to avidin (214), the amount of avidin may be calculated directly from the absorbance at 500 nm or by using the dye as an indicator in a spectrophotometric displacement from avidin with free or enzyme-bound biotin. [^{14}C]Biotin is used widely in assay systems. [^{14}C]Biotin is added to avidin and any free vitamin separated by absorping the complex onto CM-cellulose (196), Sephadex gel (204), or precipitating it with antibody (215). The unabsorbed [^{14}C]biotin can then be determined. A disadvantage of such methods is that other biotin-binding factors, or endogenous biotin present in the tissue samples, will compete with [^{14}C]biotin. This problem can be overcome by methods such as radioimmunoassay of avidin with ^{125}I (216).

The possible contribution of avidin to the antimicrobial defense of eggs is considered under Integrated Working of the Chemical Defense.

Ovoflavoprotein

All of the riboflavin of hens' egg white occurs as the flavin moiety of a flavoprotein; 1 mol of riboflavin is bound to 1 mol of apoprotein with a marked diminution of the former's capacity to fluoresce at 500 nm (217, 218). In this respect egg white flavoproteins differ from most other naturally occurring ones which have FMN or FAD as the flavin moiety (219). The riboflavin-containing flavoproteins in the serum of hens (220) and egg yolk (221, 222) are serologically identical to that present in the albumen (223).

The apoprotein, a glycoprotein with a molecular weight of 32,000 (224), has a large number of aspartate and glutamate amino acid residues which endow the protein with a strong anionic character as demonstrated by its electrophoretic mobility and absorption onto DEAE-cellulose. The protein is highly crosslinked by eight disulfide bridges; there are no free sulfydryl groups and all of the sulfur occurs as methionine or cystine (224). The apoprotein contains 14% carbohydrate made up of mannose,

galactose, and glucosamine (224). Its content of phosphorus (0.8%) can be removed by potato acid phosphatase (218) without affecting the flavin-binding capacity.

Guanine hydrochloride (5 M) causes reversible inactivation of the protein. The $S_{20,w}$ changes from 3.05 in 0.02 M NaCl to 1.75 in 5 M GuHCl suggest fragmentation into separate polypeptide chains or unfolding of a single chain to a less symmetrical molecule (224). Reduction followed by alkylation indicates that the apoprotein consists of two subunits joined probably by two thiocovalent linkages (225). The larger unit (226) has a M_r of 24,000 g/mol and contains five of the dithio linkages.

Chemical modification of specific amino acid residues have been used in attempts to identify the residues involved in the binding site. The use of 1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) and iodine modification of histidine and tyrosine residues, respectively, did not affect the binding capacity of the apoprotein. As oxidation caused a decreased absorption at 280 nm and an increase at 330 nm it would appear that a tryptophan residue is involved in binding. The pH-activity curve (224) closely resembles a titration curve for a carboxyl group with a pK_a of approximately 3.8 thereby suggesting that a carboxylate ion is essential for binding also.

The binding of riboflavin to apoprotein is not influenced by pH; at pH 4.2, the flavoprotein is dissociated but recombines above this value. Modification of the isoalloxazine ring of riboflavin at the 3 and 9 positions decreases the binding to the apoprotein (218). This suggests that riboflavin is either bound by several sites or that the entire molecule fits into a specific structure or crevice on the protein.

The content of riboflavin in the egg white of domestic hens reflects that present in the diet (227, 228). The duck, goose, and Adelie penguin, on the other hand, do not secrete riboflavin in the egg white regardless of the presence of high levels of the vitamin in the diet (218, 229).

It has been suggested (225) that the riboflavin-binding proteins of the albumen, yolk, and serum were all under the control of a single gene. The blood flavoprotein is transferred directly to the yolk but that in the albumen must arise from de novo synthesis in the oviduct. Although the effect of the altered gene, "rd" is unknown, it has been proposed that "Rd" may function through the conversion of a riboflavin-binding protein precursor, pro-RBP, to the active compound in a similar way to that in which proinsulin and prochymotrypsin are converted to insulin and chymotrypsin, respectively.

The apoprotein of egg white can be assayed directly by titration with a standard solution of riboflavin to the first visible fluorescence with a uv light as the exciting source. Fluorescence is quenched completely until a stoichiometric amount of riboflavin is added to the apoprotein. This rapid and inexpensive method gives a sharp end point. The determination of unbound riboflavin after the addition of riboflavin to apoprotein provides an indirect method of assay. The amount of apoprotein can be calculated from the bound riboflavin. Free riboflavin can be determined spectrophotometrically at 450 nm or microbiologically using a riboflavin-requiring organisms such as *Lactobacillus casei* (230, 231).

Although the inhibition of the growth of *Streptococcus pyogenes* and *L. casei* has been demonstrated *in vitro* when 10 mol of apoprotein was present for every mole of riboflavin (218), the half-saturation of the apoprotein in the hen's egg white would in theory endow this protein with only a minor role in the egg's antimicrobial defense (142). In practice however microorganisms in egg white may not be able to obtain

sufficient vitamin in the presence of other deficiencies such as iron, biotin, simple molecules of nitrogen, and at a high pH, a topic discussed under Integrated Working of the Chemical Defense.

Ovomucoid

Ovomucoid of chicken egg white acts as a proteolytic enzyme inhibitor (232–234). The heterogeneous nature of ovomucoid was demonstrated by attempts to isolate pure material (Table I). With some of the initial preparations, the heterogeneity was due to contamination with ovoinhibitor and lysozyme (80, 235). Even when these were removed, differences in sialic acid (80, 236), carbohydrate content (80), and minor variations in amino acid composition of ovomucoid persisted (80).

Ovomucoid, a glycoprotein (M_r 28,000), accounts for 11% of the total protein content in chicken egg white. The amino acid composition of several avian ovomucoids (152, 238) resembles that of other proteolytic enzyme inhibitors in that there is no tryptophan, little if any methionine, but large amounts of cystine. The carbohydrate (up to 25% w/w) in ovomucoid contains: 1–1.5% D-galactose; 4.3–4.7% D-mannose; 12.5–15.4% 2-amino-2-deoxy-D-glucose; 0.4–4.0% sialic acid; and 6–9% total hexose. The carbohydrate moiety, which consists of three oligosaccharides, is attached to the protein via a *N*-(β-aspartyl-*N*-acetylglucosaminyl) amino link between glucosamine and asparagine (239). The role of the carbohydrate has not been established; the removal of the sialic acid by neuraminidase does not affect its inhibitory activity (240).

There are three different types of ovomucoid in avian albumen (236, 241): one inhibits trypsin only or primarily; another inhibits chymotrypsin only or primarily; and a third inhibits both (Fig. 13). The last category may be subdivided further; those that inhibit equimolar amounts of trypsin and chymotrypsin and those that inhibit 2 mol of trypsin to 1 mol of chymotrypsin. Although the binding sites for trypsin and chymotrypsin differ (236) they may or may not overlap such that the binding of one enzyme may influence the binding of the other. Chicken ovomucoid shows a marked specificity; it inhibits bovine (76) and porcine (243) but not human trypsin (229).

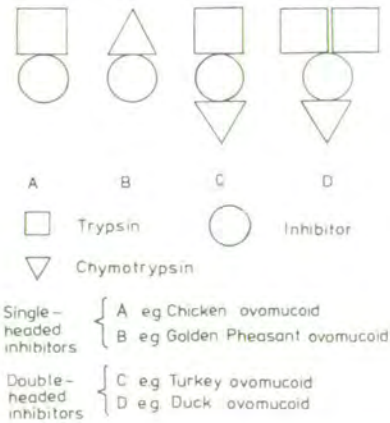

FIG. 13. Schematic diagram of the complexes formed between proteolytic enzymes and avian egg white inhibitors. Redrawn from Ref. (65).

Inhibition involves a highly associated enzyme–substrate (inhibitor) complex in which substrate recognition of a specific amino acid is considered to be the initial event. There are two types of trypsin inhibitor; those that lose their inhibitory activity upon modification of (a) their lysine or (b) their arginine residues (Table V). Thus it has been demonstrated (245) that acetylation or carbamylation of the lysine residues of turkey ovomucoid destroys its inactivation of trypsin but not of chymotrypsin. This procedure has no effect on chicken or golden pheasant ovomucoid activity against trypsin or chymotrypsin, respectively. It did however destroy the pheasants' very weak activity toward trypsin. Arginine modification with 1,2-cyclohexadione (246) led to the loss of activity of chicken ovomucoid. Removal of these substrate-like residues by treatment with carboxypeptidase B (247) eliminated activity completely. Substrate-like residues necessary for the inhibition of enzymes other than trypsin, for example chymotrypsin, have been difficult to identify because the residues involved are frequently either unreactive or the modification did not eliminate their substrate-like character.

Dissociation constants for these enzyme–inhibitor complexes are frequently less than 10^{-8} and may be as low as 10^{-13} (248). This remarkable stability led several workers to suggest that a covalent bond, formed by acylation of the enzyme's serine by the argininyl carboxyl group of the inhibitors, was the driving force and a possible catalytic intermediate in the inhibition (e.g., 249). These suggestions have been questioned by others because direct confirmation of this acyl linkage has not been possible and also because inactive derivatives of trypsin and chymotrypsin form strong specific complexes with several of the ovomucoids (e.g., 250). These observations indicate that the main strength of interaction is due to many weak noncovalent interactions such as hydrogen bonding and Van der Waals forces (251). These interactions are stabilized by a close, complementary fit corresponding to a good enzyme–substrate complex. Some covalent bonding may be involved but, contrary to earlier proposals, it is unnecessary for the formation of, and does not add to, the stability of these complexes (252).

Ovomucoid shows unusual heat stability and resistance to high concentrations of urea in neutral or acid conditions; more than 90% of its activity remained after 30 min at 80°C in 9 M urea between pH 3 and 9 but only 6% remained at pH 9 (245). Turkey and pheasant ovomucoids are even more stable to heat.

TABLE V

Combining Site Amino Acids of Avian Trypsin Inhibitors

Inhibitor	*Essential amino acid*
Chicken ovoinhibitor	Arginine
Chicken ovomucoid	Arginine
Turkey ovoinhibitor	Arginine
Turkey ovomucoid	Lysine
Cassowary ovomucoid	Lysine
Penguin ovomucoid	Lysine
Duck ovomucoid	Lysine
Quail ovoinhibitor	Arginine
Quail ovomucoid	Lysine

Ovoinhibitors

Ovoinhibitor, the other main protease inhibitor in hen egg white, was isolated by Matsushima (235) who found it inhibited bovine trypsin and proteases from *Bacillus subtilis* and *Aspergillus* spp. The similarity in structure of chymotrypsin to the bacterial protease, subtilisin, promoted studies of the effect of this inhibitor on chymotrypsin (236). This led to the observation (76) that the weak inhibitory activity against chymotrypsin reported for chicken ovomucoid was due to the presence of ovoinhibitor as a contaminant. Details of the separation of ovoinhibitor from ovomucoid are given in Table I.

Like ovomucoid, ovoinhibitor exhibits heterogeneity due primarily to the charge on the molecule which is reflected during purification with starch–gel electrophoresis or DEAE-cellulose chromatography (253, 254). The various forms are similar in their amino acid composition and antiproteolytic activity.

Chicken ovoinhibitor, a glycoprotein with a M_r of about 46,500 (253), comprises about 0.1% of the total protein in egg white. Its amino acid composition resembles that of ovomucoid (254) and its tryptophan residues are <1. There are 34 half-cystine residues and low amounts of methionine. The content of carbohydrate, as yet uncharacterized, is smaller than in ovomucoid.

Chicken ovoinhibitor is considered to be a "double-headed" inhibitor; 1 mol of inhibitor combines simultaneously with two molecules of trypsin and two of chymotrypsin (253). The absence of competition between these two is evidence that separate sites are involved. Competition is evident however when ovoinhibitor is incubated with chymotrypsin and subtilisin suggesting that the two enzymes compete for the same or closely related sites (253). Ovoinhibitor reacts with trypsins and chymotrypsins of bovine (236), avian (255), but not those of human origin (229). It differs from most ovomucoids in that arginine is the essential substrate-like residue for binding trypsin (256).

Ovoinhibitor shows appreciable stability in acid solution; 93–95% of its activity is retained during 15 min at 90°C, pH 3–5, or for 24 h at 40°C, pH 2–3, or 3 h at 40°C, pH 1. In 0.01 N NaOH, the activity is stable at 23°C for 24 h but is lost in 3 h at 40°C in 0.1 N NaOH and in 15 min at 90°C, pH 7–9 (253).

Ficin-Papain Inhibitor

A third protease inhibitor was isolated from chicken egg white by Fossum and Whitaker (257). It inhibits ficin and papain but not trypsin, chymotrypsin, or proteolytic enzymes from *Pseudomonas aeruginosa, Proteus vulgaris, Bacillus cereus*, or *Bacillus subtilis*. This inhibitor can be differentiated from ovomucoid and ovoinhibitor by CM-cellulose chromatography, lack of carbohydrate, and molecular weight (less than one-half that of ovomucoid). It is extremely heat resistant; only 10% of its activity is lost after boiling for 30 min at pH 4, however at pH 9 only 40% of the original activity remains. It reacts to form a 1:1 complex with ficin or papain; there is competition between the two enzymes probably because of common binding sites.

INTEGRATED WORKING OF THE CHEMICAL DEFENSE

The review in the preceding section showed that avian egg albumen contains many proteins that might be expected to have an adverse effect on the structural integrity or growth of microorganisms. It revealed also the extent of the information that has

been built up on the methods of isolation, modes of action and assay systems for the principal components of the albumen. In contrast there is a feeble understanding of the coordinated workings of the chemical defense offered by the albumen. This is attributable to two features of the microbiological studies which have sought to elucidate the egg's defense against infection. Far too much attention has been given to the microorganisms isolated from addled eggs and too little (128, 129) to the role of the chemical defense in protecting the embryo and food reserve during embryogenesis and perhaps in influencing the microbial colonization of the gut of the newly hatched chick.

The Role of Ovotransferrin

It is generally assumed that avian eggs are germ free at oviposition (7) and that the shell acquires a heterogeneous flora by contact with nesting materials etc. This flora is dominated by Gram-positive bacteria (258, 259). The contents of rotten eggs harbor a mixed population of Gram-negative bacteria (258, 260–263). As the agencies (see under Physical Defense) that cause flooding of the pore canals in the shell (Fig. 1) cannot be expected to have any selective action whatsoever on the composition of the flora translocated along the pore canals and deposited on the shell membranes, it has to be assumed that the outgrowth of Gram-negative bacteria from a flora dominated by Gram-positive ones must occur in the shell membranes and/or the albumen. It needs to be stressed that the initial infection remains confined to the shell membranes for 10–20 days (47, 73, 266–270) in eggs stored at 10°C or more. It would seem reasonable to assume that detailed studies of this initial phase of infection might well provide a critical means of assessing the integrated workings of the defense system of the albumen. As yet, however, it has been studied with a mixed infection on one occasion only (46). Two phases of selection of Gram-negative bacteria were noted; a range of these organisms achieved numerical dominance over the Gram-positive ones during the time that the infection was confined to the shell membranes but only particular strains of the selected flora achieved dominance in the second phase which triggered off heavy contamination of the albumen. Temperatures of incubation influenced selection also; coliform organisms became dominant at 30°C and above but pseudomonads dominated at lower temperatures. The investigators concluded that growth rate was probably the principal selective agent. Until a study such as this is repeated with the organisms being characterized not only for purposes of identification but also on the basis of nutritional requirements, the possible contribution of avidin, the apoprotein that combines with riboflavin, and maybe even the protease inhibitors, cannot be assessed.

When organisms isolated from rotten eggs are used to study the antimicrobial defense of the albumen, it ought to be appreciated, but rarely is, that, through stringent selection, they may have attributes, both constitutive and inducible, that favored their selection in the harsh environment of the albumen. Indeed it may well be that only one of the factors of the albumen plays an important role in the control of their growth. The literature (271) supports the view that ovotransferrin is the cardinal factor. The results presented in Fig. 14 exemplify those used by many workers to arrive at this conclusion. Likewise the results given in Fig. 15 not only support this conclusion but also the assertions by Haines (6) and Brooks (41) that the content of simple nitrogenous compounds in the albumen is too low to support maximal growth

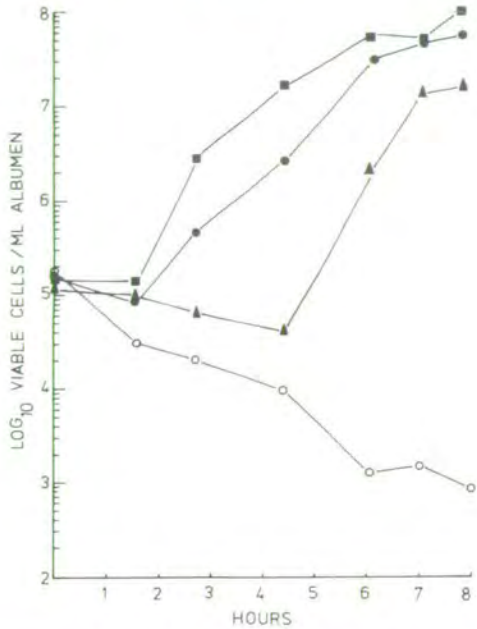

FIG. 14. The effect of iron salts on the death of *Escherichia coli* 0141 in hen egg white (pH 9.2; incubation temperature 39.5°C). Solid triangle, $FeCl_3$; solid circle, $FeSO_4$; solid square, $Fe(NH_4)_2(SO_4)_2 . 6H_2O$, and open circle, unsupplemented albumen (H. S. Tranter, unpublished observations).

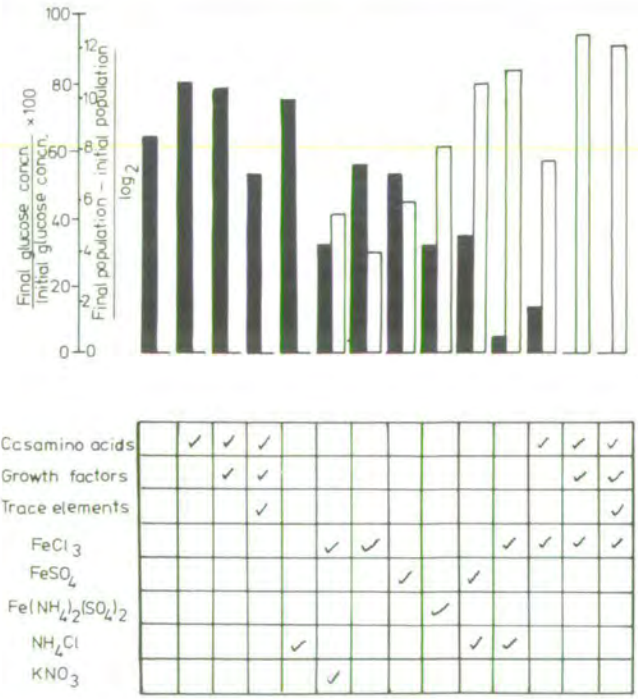

FIG. 15. The influence of various additives on growth of (open bars) and glucose utilization (solid bars) by *Escherichia coli* C20 in hen egg white (pH 9.2) at 39.5°C (H. S. Tranter, unpublished observations).

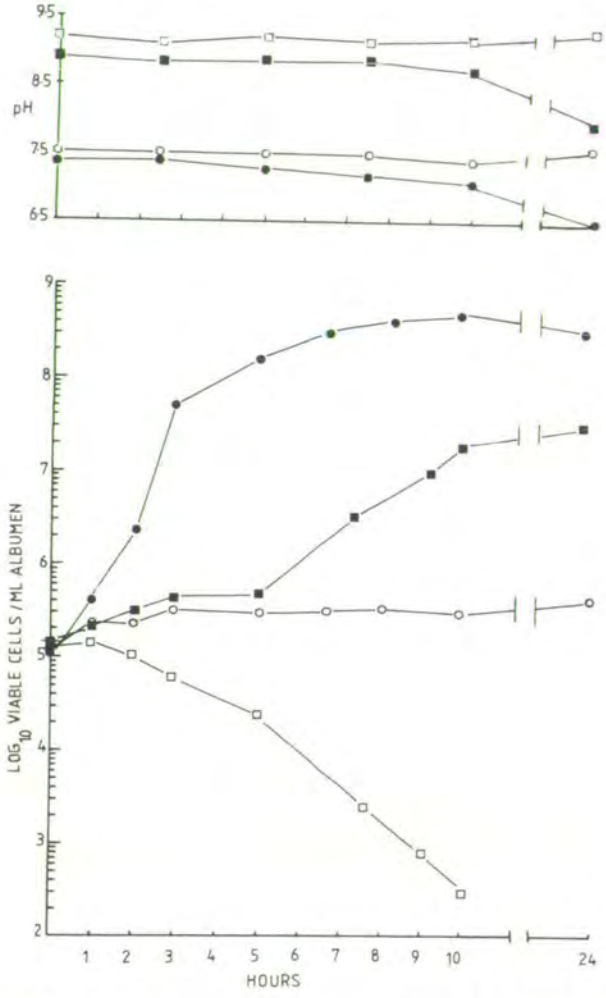

FIG. 16. The effect of pH on the behavior of *Escherichia coli* 0111 in unsupplemented (open symbols) and Fe^{3+}-saturated egg white (solid symbols) at 39.5°C (H. S. Tranter, unpublished observations).

of nonfastidious microorganisms such as coliforms or pseudomonads. Our studies have shown that pH influences the response of microorganisms to albumen (Fig. 16) and that the temperature of incubation determines the fate of microorganisms deprived of Fe^{3+} by ovotransferrin (Fig. 17). Thus bacteriostasis was a common feature of iron-deprived bacteria incubated at or below the midpoint of the temperature range for their growth whereas death occurred at temperatures at the upper end of the range. It is pertinent to note that transferrin of mammalian serum acts, both *in vivo* and *in vitro*, as a bacteriostatic agent (e.g., 272–274); the iron-deprived bacteria may die from lysis caused by complement and probably lysozyme also. The occurrence of the former in egg albumen has not been reported and we were unable to demonstrate lysozyme-induced lysis of bacteria denied Fe^{3+} (Fig. 11). Studies of the antimicrobial properties of polymorphonuclear lymphocytes of rabbits have revealed (275, 276) that cationic proteins of these cells kill bacteria. As such proteins are

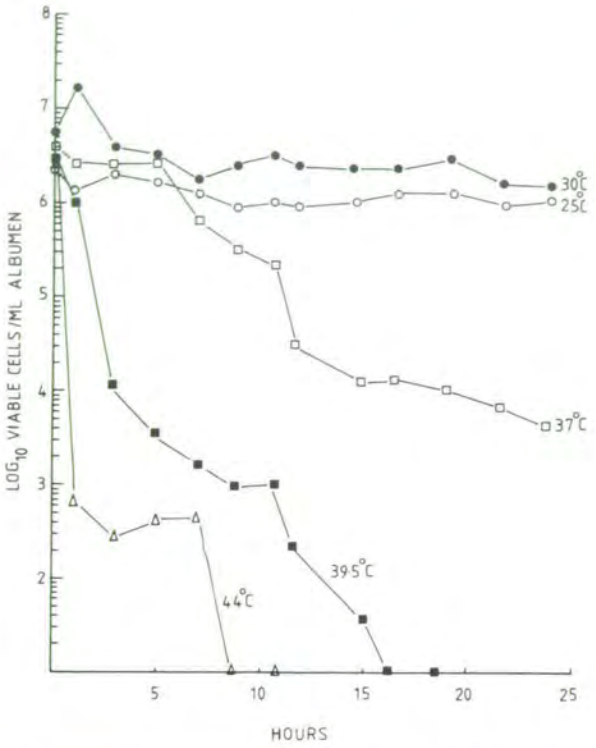

FIG. 17. The influence of incubation temperature on the behavior of *Escherichia coli* C3650 in hen egg white (H. S. Tranter, unpublished observations).

precipitated when iron is used to quench the lactoferrin, and hence the bacteriostatic action of the leucocytes, it could be that the death of bacteria in egg white might be due to an as yet uncharacterized protein of the albumen.

An Inducible Defense against Ovotransferrin?

In general microbiologists have failed to appreciate fully the problems which confront iron-requiring microrganisms whose natural niche has a neutral pH and a pO_2 in equilibrium with the atmosphere. Under such conditions Fe(II) will be oxidized to the Fe(III) state which in turn is readily hydrolyzed to form insoluble ($K_{sp} < 10^{-38}$ mol/liter) high-molecular-weight polymers of Fe(III) oxyhydroxide (277) by

$$Fe(H_2O)_6^{3+} \rightleftharpoons Fe(H_2O)_5(OH)^{2+} + H^+,$$

$$Fe(H_2O)_5(OH)^{2+} \rightleftharpoons Fe(H_2O)_4(OH)_2^+ + H^+,$$

$$Fe(H_2O)_4(OH)_2^+ \rightleftharpoons Fe_2(H_2O)_8(OH)_2^{2+} + H^+.$$

Subsequent steps lead to the formation of the spherical polymers described in (277). Thus microorganisms that need only modest amounts of Fe^{3+} (0.3–4.0 μM/bacterium; (278)) have evolved specific molecules for the scavenging (Table VI), transport (Fig. 18), and storage of this element. Organisms, such as *Arthrobacter terregens*, coexist with microorganisms having the first-mentioned attribute. Similarly iron-transport

TABLE VI

Microbial Siderophores

Compound	Ligand system: Type	Ligand system: No./mol	Source
Enterobactin (enterochelin)	Catechol	3	*Aerobacter aerogenes Escherichia coli Salmonella typhimurium Klebsiella pneumoniae Shigella sonnei*
2,3-Dihydroxy-*N*-benzoyl-L-serine	Catechol	1	As above
2,3-Dihydroxy-*N*-benzoylglycine	Catechol	1	*Bacillus subtilis*
Agrobactin	Catechol Spermidine Threonine	3 1 1	*Agrobacterium tumefaciens*
Pyochelin	Catechol-like	NA	*Pseudomonas aeruginosa*
Aerobactin	Hydroxamic acid Carboxylic acid Alcoholic hydroxyl	2 1 1	*Aerobacter aerogenes*
Schizokinen	Hydroxamic acid Carboxylic acid Alcoholic hydroxyl	2 1 1	*Bacillus megaterium*
Mycobactins	Hydroxamic acid Phenolic hydroxyl Tertiary N	2 1 1	*Mycobacterium smegmatis Mycobacterium tuberculuosis Mycobacterium kansasii*
Ferrichromes	Hydroxamic acid	3	*Aspergillus, Neurospora, Penicillium, Ustilago, Actinomyces, Streptomyces, Cryptococcus* spp.
Rhodotorulic acid	Hydroxamic acid	2	*Rhodotorula, Leucosporidium, Sporobolomyces* spp.
Fusarinines	Hydroxamic acid	1–3	*Fusaria, Aspergillus, Gibberella* spp.
Ferrioxamines	Hydroxamic acid		*Streptomyces, Nocardia* spp.
$Pyoverdine_{pf}$	Hydroxamic acid-like	NA[a]	*Pseudomonas fluorescens*

[a] Value not available at the present time.

proteins, the transferrins, ensure that Fe(III) oxyhydroxides do not form in the circulating fluids of animals (140), the actual amount of free iron in equilibrium with these proteins is of the order of 10^{-18} M. Thus the results presented in Figs. 14 and 15 can be taken as evidence that this level of free iron is too low to support the growth of *Escherichia coli.*

If we turn to the literature concerned with microbial growth in mammalian serum *in vitro*, then three reactions of microorganisms to iron deprivation can be listed: some bacteria are rapidly killed (272), some grow normally (279), and others grow

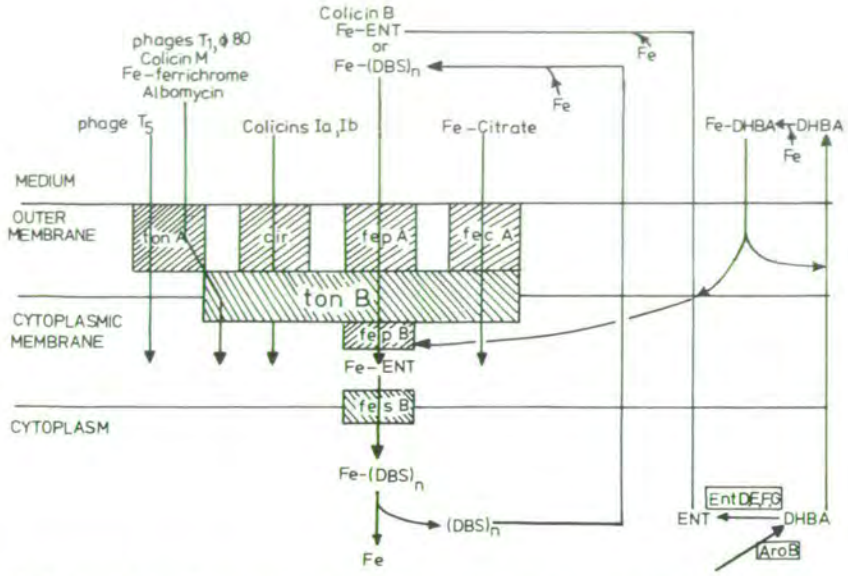

FIG. 18. Receptor-dependent translocation of iron across the outer membrane of *Escherichia coli* K-12. The figure shows the common binding sites of bacteriophages, colicins, and ferri-siderophores in the outer membrane. The proteins coded for by the genes *tonA*; *cir*, *fepA*; *fecA* have been identified and located in the outer membrane. Translocation processes requiring the *tonB* function are shown by an arrow passing through the area designated *tonB*. Possible pathways for the transport of ferric-enterobactin, ferric-$(DBS)_n$, and ferric-DHBA across the membrane and the release of iron into the cytoplasm are shown. The proposed sites of action of the various gene products are indicated by the inclusion of the gene mnemonic in a box. Adapted from Ref. (237, 242, 244).

to a limited extent only (279). As was noted previously, rapid killing has been associated with complement-induced lysis of the bacterial cell. Bacteria that have a requirement for Fe^{3+} and grow rapidly must be adapted so that they scavenge sufficient of this element for their needs. Some, for example *Pasteurella pestis*, may be able to interact (280) with transferrin in a manner analogous to that of reticulocytes (281) but the majority probably secrete compounds (Figs. 19, 20) that remove iron from

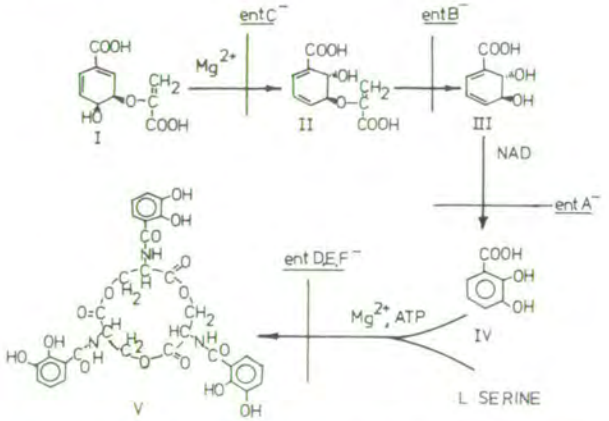

FIG. 19. The biosynthesis of enterobactin. **I.** Chorismate; **II.** isochorismate; **III.** 2,3-dihydro-2,3-dihydroxybenzoic acid; **IV.** DHBA; **V.** enterobactin. The figure shows the reactions affected by mutations in the six *ent* genes. From (264).

Compound	Structure
Ferrichrome	R=R'=R''=H;R'''=CH_3-
Ferrichrome A	R=R'=$HOCH_2$;R''=H
Ferrichrome C	R''' = CH$_3$ / H trans / CH_2COOH
Ferrichrysin	R=R''=H
Ferricrocin	R'=R'''=CH_3-(2)
	R=R'=$HOCH_2$-;R''=H;
	R'''=CH_3-
	R=R''=H;R'=CH_2OH;
	R'''=CH_3-.

FIG. 20. Hydroxamate siderophores; the ferrichrome family. From (265).

the transferrin molecule and then re-enter the bacterial cell (Fig. 18). Indeed it has been shown (282) that there was a direct relationship between the virulence of *Escherichia coli* and the ability to synthesize catechols, providing that a serum did not contain antibodies that blocked the synthesis of the chelating agent. As the hens' egg albumen does not appear to contain such antibodies, the question arises: can microorganisms in the albumen counter the bacteriostatic action of ovotransferrin through the synthesis of chelating agents? Garibaldi (283) reported the growth of *Salmonella typhimurium* in albumen to which iron transport compounds had been added.

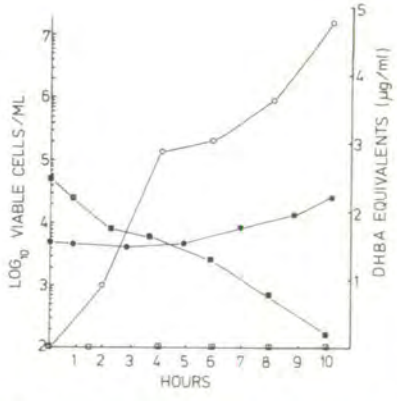

FIG. 21. The behavior of *Escherichia coli* 0141 in iron-deficient (<0.5 μg ml^{-1} iron) M9 medium, pH 7.6 (solid circles), and unsupplemented hen egg white, pH 9.2 (solid squares) at 37°C with corresponding catechol production based on equivalents of 2,3-dihydroxybenzoic acid (open symbols).

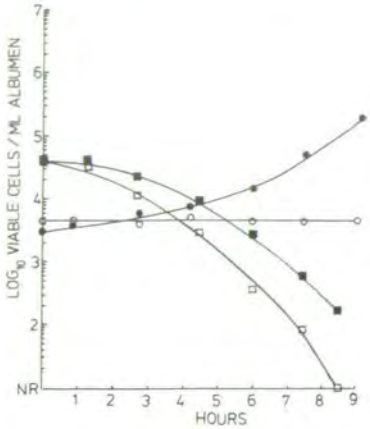

FIG. 22. The effect of purified Fe-enterobactin (10 mg ml^{-1}) on the behavior of *Escherichia coli* 0141 in hen egg white at pH 7.9 (circles) and pH 9.4 (squares). Unsupplemented albumen, open symbols; albumen plus chelate, solid symbols.

We have noted that *Escherichia coli* produced catechols in an iron-depleted mineral salts medium (incubation, 37°C) at pH 7.6 but not in albumen at 9.2 (Fig. 21). Moreover this organism grew to a limited extent in albumen supplemented with enterobactin when the pH was poised at 7.9; it was killed in supplemented albumen pH 9.4 (Fig. 22). The latter result we attributed to alkaline hydrolysis of enterobactin—the molecule is vulnerable to oxidation of the hydroxyl groups and hydrolysis of the lactone—and the failure of the breakdown products (Fig. 23), which are known to have feeble chelating potential (284, 285), to act as iron transport compounds. In a discussion of the potential of an inducible property of a microorganism to ameliorate an unfavorable component of an environment, it must be stressed that the induction of such a property may well be suppressed by another environmental factor. It is pertinent to note, therefore, that the production of iron-scavenging compounds (Table VI) by *Salmonella typhimurium, Escherichia coli*, and a fluorescent pseudomonad

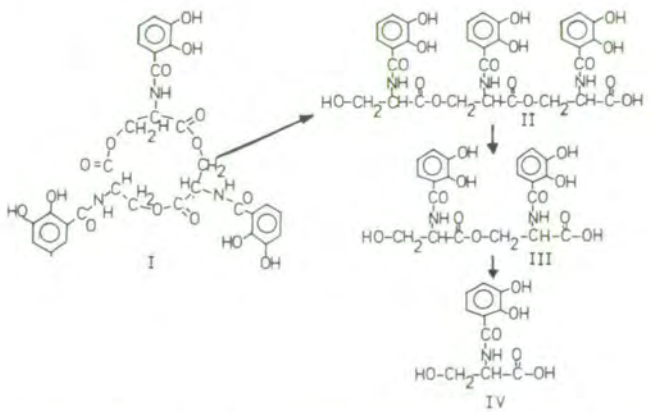

FIG. 23. Enterobactin and its hydrolytic breakdown products. **I.** Enterobactin (cyclic DBS trimer). **II.** Linear DBS trimer. **III.** DBS dimer. **IV.** DBS. Taken from (264).

is substantially diminished by a small increase above the optimum temperature for growth (286–288).

A discussion of the question—is there an inducible defense that protects microorganisms against bacteriostasis due to iron-deprivation by ovotransferrin—has in practice thrown a new light on the integrated workings of the various components of the albumen. In practice it has shown that, at least with *Escherichia coli*, ovotransferrin operates along with pH (not only does the alkaline reaction stop dissociation of Fe^{3+}-ovotransferrin but it hydrolyses entrobactin also), the low levels of simple nitrogenous compounds, and incubation temperature in the defense of eggs against microbial infection. Of these, the influence of temperature is likely to be the most variable. In nature, for example, the albumen of an egg will be at ambient temperature until a clutch is completed and incubation begins. An extensive study of the environment of the nest cup with sophisticated monitoring equipment revealed (289) that there are pronounced temperature variations in the nest cup throughout incubation—a situation that is in marked contrast to the stringent temperature control in commercial incubators.

When discussing the results obtained from an extensive study of bacterial rotting of washed eggs, Gillespie and Scott (73) noted "many of the eggs infected with *Pseudomonas* species were opened at an early stage when the infection had not become general and, in some of these, the bacterial growth was confined to a small bright green patch in the albumen." We too have seen such patches (51) in eggs and deduced that these represented the albumen that was present at the junction of the yolk and infected shell membranes at the time when the second phase of microbial infection

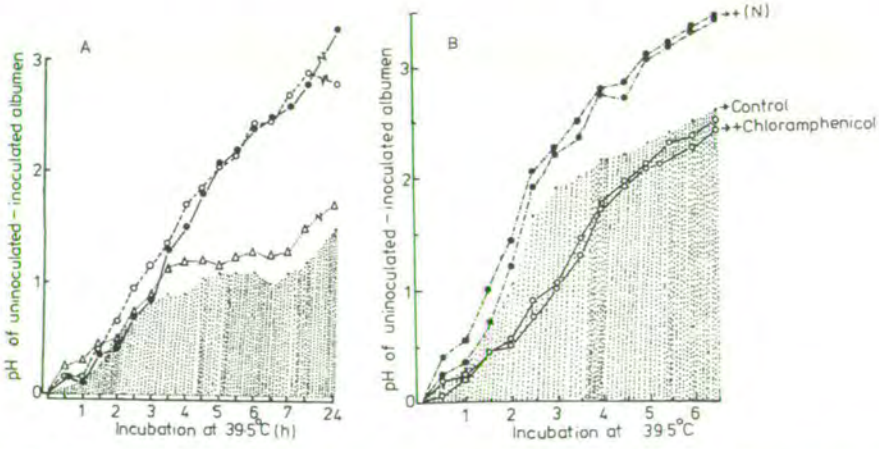

FIG. 24. (A) The effect of additives on the acid drift in albumen seeded with a heavy suspension of *Escherichia coli*. Beakers containing 50 ml of albumen and equivalent to 162 μg dry wt *E. coli*/ml and covered with aluminum foil were held in a water bath at 39.5°C. Initial pH of albumen, 8.7. Shaded area, inoculated albumen alone; open triangles, inoculated albumen containing ferric chloride (8 μM); open circles, inoculated albumen containing ferrous ammonium sulfate (8 μM); and solid circles, inoculated albumen containing amonium chloride (16 μM). (B) The effect of chloramphenicol on the acid drift in albumen seeded with a heavy suspension of *Escherichia coli* (equivalent to 413 μg dry wt/ml). Initial pH of albumen, 9.05. Shaded area, inoculated albumen; solid circles, the range of readings obtained at 30-min intervals with seeded albumen containing ferrous ammonium sulfate (8 μM), ferric chloride (8 μM), or ferric chloride and ammonium chloride (8 μM; 16 μM), and open circles, the range of readings obtained with supplemented albumen containing chloramphenicol (20 mg/ml). Taken from (293).

leading to generalized infection of the albumen was beginning. It has been shown, also, that the metabolism of a facultative anaerobe, *Staphylococcus aureus*, is changed from respiration to glycolysis in a synthetic medium containing ovotransferrin (290, 291). Another adaptation to counter the action of ovotransferrin is suggested by the observation that a clump of fermentative bacteria would slowly increase in size until the whole of the albumen was colonized. In practice we have been unable to demonstrate any differences in response when *Escherichia coli* grown anaerobically or aerobically were used to seed albumen. Moreover, the addition of very large inocula of this organism to albumen did not lead to rapid fermentation of the glucose (292, 293), as indexed by change in pH (Fig. 24). Indeed it is worthy of note that rapid fermentation only occurred when the albumen was supplemented with a source of combined nitrogen and that the influence of the supplement was negated by chloroamphenicol. In this type of experiment, iron supplements had only a small affect on the rate of fermentation.

The evidence and concepts discussed above indicate that a complex antimicrobial defense is present in egg albumen; as it functions in the absence of a vascular system or neural or hormonal control, it would seem reasonable to identify it with the systems which Kochan (294) referred to as "nutritional immunity."

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REFERENCES

1. PRICE, P. W. (1974) *Evolution* **28,** 76–84.
2. LACK, D. (1954) The Natural Regulation of Animal Numbers, Clarendon, Oxford.
3. CODY, M. L. (1971) *in* Avian Biology (Farner, D. S., and King, J. R., eds.), Vol. 1, pp. 461–512, Academic Press: New York.
4. NEEDHAM, J. (1931) *in* Chemical Embryology, Cambridge Univ. Press, Cambridge.
5. CALDER, W. A. (1979) *Bioscience* **29,** 461–467.
6. HAINES, R. B. (1939) Rep. Food Invest., Vol. 47, HMSO, London.
7. BROOKS, J., AND TAYLOR, D. I. (1955) Rep. Food Invest., Vol. 60, HMSO, London.
8. MILLER, W. A., AND CRAWFORD, L. B. (1953) *Poult. Sci.* **32,** 303–309.
9. BROWN, W. E., BAKER, R. C., AND NAYLOR, H. B. (1966) *Poult. Sci.* **45,** 284–287.
10. TYLER, C. (1953) *J. Sci. Food Agric.* **4,** 266–272.
11. ROMANOFF, A. L., AND ROMANOFF, A. J. (1949) The Avian Egg, Wiley, New York.
12. TYLER, C. (1956) *J. Sci. Food Agric.* **7,** 483–493.
13. HAINES, R. B., AND MORAN, T. (1940) *J. Hyg.* **40,** 453–461.
14. BOARD, R. G., AND HALLS, N. A. (1973) *Brit. Poult. Sci.* **14,** 69–97.
15. BOARD, R. G., AND HALLS, N. A. (1973) *Brit. Poult. Sci.* **14,** 311–314.
16. BOARD, R. G. (1974) *Brit. Poult. Sci.* **15,** 415–419.
17. BOARD, R. G. (1975) *Brit. Poult. Sci.* **16,** 89–91.
18. TULLETT, S. G., LUTZ, P. L., AND BOARD, R. G. (1975) *Brit. Poult. Sci.* **16,** 93–95.
19. BOARD, R. G. (1982) *Biol. Rev.* **57,** 1–28.
20. OREL, V. (1959) *Poult. Sci.* **38,** 8–12.
21. PATON, A. M., AND AYRES, J. C. (1964) *Nature (London)* **204,** 803–804.
22. BOARD, P. A., AND BOARD, R. G. (1967) *Lab. Pract.* **16,** 471–482.
23. BOARD, R. G. (1980) *J. Appl. Bacteriol.* **48,** 303–313.
24. TYLER, C. (1961) *J. Sci. Food Agric.* **12,** 470–475.
25. LIFSHITZ, A., AND BAKER, R. C. (1964) *Poult. Sci.* **43,** 527–528.
26. BELLAIRS, R., AND BOYDE, A. (1969) *Z. Zellforsch. Mikrosk. Anat.* **96,** 237–249.
27. MASSHOLF, W., AND STOLPMANN, H. J. (1961) *Z. Zellforsch. Mikrosk. Anat.* **55,** 818–832.
28. BAKER, J. R., AND BALCH, D. A. (1962) *Biochem. J.* **82,** 352–361.
29. STARCHER, B. C., AND KING, G. S. (1980) *Connect. Tissue Res.* **8,** 53–55.
30. HARRIS, E. D., BLOUNT, J. E., AND LEACH, R. M. (1980) *Sciences (N. Y.)* **208,** 55–56.

31. LEACH, R. M. (1978) *Poult. Sci.* **57,** 1151.
32. LEACH, R. M., RUCKER, R. B., AND VAN DYKE, G. P. (1981) *Arch. Biochem. Biophys.* **207,** 353–359.
33. ROBINSON, D. S., AND KING, N. R. (1968) *J. R. Microsc. Sci.* **88,** 13–22.
34. COOKE, A. S., AND BALCH, D. A. (1970) *Brit. Poult. Sci.* **11,** 345–352.
35. WALDEN, C. C., ALLEN, I. V. F., AND TRUSSELL, P. C. (1956) *Poult. Sci.* **35,** 1190–1196.
36. GARIBALDI, J. A., AND STOKES, J. L. (1958) *Food Res.* **23,** 283–290.
37. BOARD, R. G. (1965) *J. Appl. Bacteriol.* **28,** 437–453.
38. BEAN, K. C., AND MACLAURY, D. W. (1959) *Poult. Sci.* **38,** 693–698.
39. WILLIAMS, J. E., AND WHITTEMORE, A. D. (1967) *Avian Dis.* **11,** 467–490.
40. WHITE, A., HANDLER, P., AND SMITH, E. L. (1973) Principles of Biochemistry, McGraw–Hill, New York.
41. BROOKS, J. (1960) *J. Appl. Bacteriol.* **23,** 495–509.
42. BOARD, R. G., AND AYRES, J. C. (1965) *Appl. Microbiol.* **13,** 358–364.
43. KOROTKOVA, G. P. (1957) *J. Gen. Chem. USSR* **17,** 275–285.
44. BOARD, R. G. (1966) *J. Appl. Bacteriol.* **29,** 319–341.
45. STUART, L. S., AND MCNALLY, E. H. (1943) *U. S. Egg Poult. Mag.* **49,** 28–47.
46. SEVIOUR, E. M., AND BOARD, R. G. (1972) *Brit. Poult. Sci.* **13,** 33–43.
47. ZAGAEVSKY, J. S., AND LUTIKOVA, P. O. (1944) *U. S. Egg Poult. Mag.* **50,** 17–123.
48. STOKES, J. L., OSBORNE, W. W., AND BAYNE, H. G. (1956) *Food Res.* **21,** 510–518.
49. GARIBALDI, J. A., AND BAYNE, H. G. (1962) *J. Food Sci.* **27,** 57–59.
50. DAVIS, B. D., DULBECCO, R., EISEN, H. N., AND GINSBERG, H. S. (1975) Microbiology, Harper & Row, Hagerstown, Md.
51. BOARD, R. G. (1964) *J. Appl. Bacteriol.* **27,** 350–364.
52. BOARD, P. A., HENDEN, L. P., AND BOARD, R. G. (1968) *Brit. Poult. Sci.* **9,** 211–215.
53. POWRIE, W. D. (1977) *in* Egg Science and Technology (Stadelman, W. J., and Cotterill, O. J., eds.), pp. 65–91, AVI, Westport, Conn.
54. WURTZ, R. (1890) *Semin. Med. Paris* **3,** 21.
55. LASCHTSCHENKO, P. (1909) *Z. Hyg. Infektionskr.* **64,** 419.
56. RETTGER, L. F., AND SPERRY, J. A. (1912) *J. Med. Res.* **26,** 55–64.
57. PARASCANDOLE, C. (1893) *Centralbl. Bakteriol.* **14,** 219.
58. FLEMING, A. (1922) *Proc. R. Soc. B* **93,** 306–317.
59. HEALEY, D. J., AND PETER, A. M. (1925) *Amer. J. Physiol.* **74,** 363–368.
60. SHARP, P. F., AND WHITAKER, R. (1927) *J. Bacteriol.* **14,** 17–46.
61. FRIEDBERGER, E., AND HODER, F. (1932) *Z. Immunitaetsforsch Exp. Ther.* **74,** 429–447.
62. SCHADE, A. L., AND CAROLINE, L. (1944) *Sciences (N. Y.)* **100,** 14–15.
63. ALDERTON, G., WARD, W. H., AND FEVOLD, H. L. (1946) *Arch. Biochem.* **11,** 9–13.
64. FEENEY, R. E., AND KOMATSU, S. K. (1967) *Struct. Bonding* **1,** 149–206.
65. FEENEY, R. E., AND ALLISON, R. G. (1969) Evolutionary Biochemistry of Proteins, Wiley-Interscience, New York.
66. FRAENKEL-CONRAT, H., AND FEENEY, R. E. (1950) *Arch. Biochem.* **29,** 101–113.
67. FEENEY, R. E., AND NAGY, D. A. (1952) *J. Bacteriol.* **64,** 629–643.
68. DUCAY, E. D., KLINE, L., AND MANDELES, S. (1960) *Poult. Sci.* **39,** 831–835.
69. ELLIOTT, L. E., AND BRANT, A. W. (1957) *Food Res.* **22,** 241–250.
70. HARTUNG, T. E., AND STADELMAN, W. J. (1963) *Poult. Sci.* **42,** 147–150.
71. TRANTER, H. S., SPARKS, N. H. C., AND BOARD, R. G. (1983) Submitted for publication.
72. BROOKS, J., AND HALE, H. P. (1959) *Biochim. Biophys. Acta* **32,** 237–250.
73. GILLESPIE, J. H., AND SCOTT, W. J. (1950) *Aust. J. Appl. Sci.* **1,** 514–530.
74. LUSH, I. E. (1961) *Nature (London)* **189,** 981–984.
75. BAKER, C. M. A., AND MANWELL, C. (1962) *Brit. Poult. Sci.* **3,** 161–174.
76. FEENEY, R. E., ABPLANALP, H., CLARY, J. J., EDWARDS, D. L., AND CLARK, J. R. (1963) *J. Biol. Chem.* **238,** 1732–1736.
77. OGDEN, A. L., MORTON, J. R., GILMOUR, D. G., AND MCDERMID, E. M. (1962) *Nature (London)* **195,** 1026–1028.
78. FERGUSON, A. (1971) *Comp. Biochem. Physiol.* **38B,** 477–486.
79. FRELINGER, J. A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 326–329.
80. FEENEY, R. E., OSUGA, D. T., AND MAEDA, H. (1967) *Arch. Biochem. Biophys.* **119,** 124–132.
81. DAVIES, R. C., NEUBERGER, A., AND WILSON, B. M. (1969) *Biochim. Biophys. Acta* **178,** 294–303.

82. SIBLEY, C. G. (1960) *Condor* **59,** 166–191.
83. SIBLEY, C. G. (1970) Bulletin No. 32, Peabody Museum of Natural History, Yale University.
84. OSUGA, D. T., AND FEENEY, R. E. (1974) *in* Toxic Constituents of Animal Foodstuffs (Liener, I. E., ed.), pp. 39–71, Academic Press, New York.
85. SALTON, M. R. J. (1952) *Nature (London)* **170,** 746–747.
86. WEIBULL, C. (1953) *J. Bacteriol.* **66,** 688–695.
87. ZINDER, N. D., AND ARNDT, W. F. (1956) *Proc. Nat. Acad. Sci. USA* **42,** 586–590.
88. BERGER, L. E., AND WEISER, R. S. (1957) *Biochim. Biophys. Acta* **26,** 517–521.
89. SALTON, M. R. J., AND GHUYSEN, J. M. (1959) *Biochim. Biophys. Acta* **36,** 552–554.
90. ARAKI, Y., NAKATANI, T., NAKAYAMA, K., AND ITO, E. (1972) *J. Biol. Chem.* **247,** 6312–6322.
91. AMANO, K., ARAKI, Y., AND ITO, E. (1980) *Eur. J. Biochem.* **107,** 547–553.
92. ROGERS, H. J., AND PERKINS, H. R. (1968) Cell Wall Membranes, Spon, London.
93. VOS, J. C. (1964) *J. Gen. Microbiol.* **35,** 313–317.
94. KOHN, A. (1960) *J. Bacteriol.* **79,** 697–706.
95. BIRDSELL, D. C., AND COTA-ROBLES, E. H. (1967). *J. Bacteriol.* **93,** 427–437.
96. JOLLÈS, J., JAUREGUI-ADELL, J., BERNIER, I., AND JOLLÈS, P. (1963) *Biochim. Biophys. Acta* **78,** 668–689.
97. CANFIELD, R. E. (1963) *J. Biol. Chem.* **238,** 2698–2707.
98. BROWN, J. R. (1964) *Biochem. J.* **92,** 13P.
99. JOHNSON, L. N., AND PHILLIPS, D. C. (1965). *Nature (London)* **206,** 761–763.
100. BLAKE, C. C. F., KOENIG, D. F., MAIR, G. A., NORTH, A. C. T., PHILLIPS, D. C., AND SARMA, V. E. (1965) *Nature London)* **206,** 757–761.
101. PHILLIPS, D. C. (1966) *Sci. Amer.* **215,** 78–90.
102. PHILLIPS, D. C. (1967) *in* Proceedings, 7th International Congress on Biochemistry, Tokyo.
103. KOSHLAND, D. (1958) *Proc. Nat. Acad. Sci. USA* **44,** 98–104.
104. RUPLEY, J. A. (1967) *Proc. R. Soc. B* **167,** 416–428.
105. SHARON, N., AND SEIFTER, S. (1964) *J. Biol. Chem.* **239,** 2398–2399.
106. KRAVCHENKO, N. A. (1967) *Proc. R. Soc. B* **167,** 429–430.
107. PARRY, R. M., CHANDAN, C., AND SHAHANI, K. M. (1965) *Proc. Soc. Exp. Biol. Med.* **119,** 384–386.
108. CHANG, K. Y., AND CARR, C. W. (1971) *Biochim. Biophys. Acta* **229,** 496–503.
109. SAINT-BLANCARD, J., CHUZEL, P., MATHIEU, Y., PERROT, J., AND JOLLÈS, P. (1970) *Biochim. Biophys. Acta* **220,** 300–306.
110. JOLLÈS, J., SCHOENIGEN, F., JOLLÈS, P., PRAGER, E. M., AND WILSON, A. C. (1976) *J. Mol. Evol.* **8,** 59–78.
111. JOLLÈS, P., SEN, A., PRAGER, E. M., AND JOLLÈS, J. (1962) *J. Mol. Evol.* **10,** 261–264.
112. MANWELL, C., AND BAKER, C. M. A. (1973) *Ibis* **115,** 586–589.
113. CANFIELD, R. E., AND MCMURRY, S. (1967) *Biochem. Biophys. Res. Commun.* **26,** 38–42.
114. JOLLÈS, P., SAINT-BLANCARD, J., CHARLEMAGNE, D., DIANOUX, A-C., JOLLÈS, J., AND LE BARON, J-L., (1968) *Biochim. Biophys. Acta* **151,** 532–534.
115. ARNHEIM, N., INOUYE, M., LAW, L., AND LAUDIN, A. (1973) *J. Biol. Chem.* **248,** 233–236.
116. GEOFFROY, P., AND BAILEY, C. J. (1975) *Biochem. Soc. Trans.* **3,** 1212–1214.
117. BOTT, R., AND SARMA, R. (1975) *Acta Crystallogr. Sect. A* **31,** 525.
118. ARNHEIM, N., AND STELLER, R. (1970) *Arch. Biochem. Biophys.* **141,** 656–661.
119. DIANOUX, A-C., AND JOLLÈS, P. (1967) *Biochim. Biophys. Acta* **133,** 472–479.
120. CHARLEMAGNE, D., AND JOLLÈS, P. (1967) *Bull. Soc. Chim. Biol.* **49,** 1103–1113.
121. WILSON, A. C., AND PRAGER, E. M. (1974) *in* Lysozyme (Osserman, E. F., Canfield, R. E., and Beychok, S., eds.), pp. 127–141, Academic Press, New York.
122. ARNON, R., TEICHER, E., AND SCHERAGA, H. A. (1974) *J. Mol. Biol.* **90,** 403–407.
123. IBRAHIMI, I. M., PRAGER, E. M., WHITE, T. J., AND WILSON, A. C. (1979) *Biochemistry* **18,** 2736–2744.
124. MORGAN, F. J., AND ARNHEIM, N. (1974) *in* Lysozyme (Osserman, E. F., Canfield, R. E. and Beychok, S., eds.), pp. 81–88, Academic Press, New York.
125. ARNHEIM, N. (1974) *in* Lysozyme (Osserman, E. F., Canfield, R. E., and Beychok, S., eds.), pp. 153–161, Academic Press, New York.
126. HINDENBURG, A., SPITZRAGEL, J., AND ARNHEIM, N. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 1653–1657.
127. GARIBALDI, J. A. (1960) *Food Res.* **25,** 337–344.

128. BOARD, R. G., AND FULLER, R. (1974) *Biol. Rev.* **49,** 15–49.
129. TOKIN, B. P. (1964) *Folia Biol. (Prague)* **10,** 61–70.
130. MOVCHAN, N. A. (1964) *Vestnik. Leninger. Univ. Ser. Biol.* **3,** 54–65.
131. HAWTHORNE, J. R. (1950) *Biochim. Biophys. Acta* **6,** 94–96.
132. DAM, R. (1971) *Poult. Sci.* **50,** 1824–1831.
133. COTTERILL, O. J., AND WINTER, A. R. (1955) *Poult. Sci.* **34,** 679–686.
134. ROBINSON, D. S., AND MONSEY, J. B. (1971) *Biochem. J.* **121,** 537–547.
135. ROBINSON, D. S., AND MONSEY, J. B. (1972a) *J. Sci. Food Agric.* **23,** 29–38.
136. ROBINSON, D. S., AND MONSEY, J. B. (1975) *Biochem. J.* **147,** 55–62.
137. KATO, A., IMOTO, T., AND YAGASHITA, K. (1975) *Agric. Biol. Chem.* **89,** 541–544.
138. SAUTER, E. A., AND PETERSEN, C. F. (1972) *Poult. Sci.* **51,** 957–960.
139. GOEL, V. K., VENUGOPALAN, C. S., AND VERMA, S. S. (1975) *Ind. J. Anim. Sci.* **45,** 570–571.
140. ZSCHOCKE, R. H., AND BEZKOROVAINY, A. (1974) *Drug Res.* **24,** 726–737.
141. MASSON, P. L., AND HEREMANS, J. F. (1971) *Comp. Biochem. Physiol.* **39B,** 119–129.
142. BAKER, C. M. A. (1968) *in* Egg Quality: A Study of the Hen's Egg (Carter, T. C., ed.), pp. 67–108, Oliver & Boyd, Edinburgh.
143. CLARK, J. R., OSUGA, D. T., AND FEENEY, R. E. (1963) *J. Biol. Chem.* **238,** 3621–3631.
144. AISEN, P., LEIBMAN, A., AND REICH, H. A. (1966) *J. Biol. Chem.* **241,** 1666–1671.
145. AASA, R., MALMSTROM, B. G., SALTMAN, P., AND VANNGARD, T. (1963) *Biochim. Biophys. Acta* **75,** 203–222.
146. SCHADE, A. L., REINHART, R. W., AND LEVY, H. (1949) *Arch. Biochem.* **20,** 170–172.
147. HOLMBERG, C. G., AND LAURELL, C. B. (1947) *Acta Chem. Scand.* **1,** 944–950.
148. WARNER, R. C., AND WEBER, I. (1953) *J. Biol. Chem.* **75,** 5094–5101.
149. TAN, A. T., AND WOODWORTH, R. C. (1969) *Biochemistry* **8,** 3711–3716.
150. GABER, B. P., AND AISEN, P.(1970) *Biochim. Biophys. Acta* **221,** 228–233.
151. BATES, G. W., WORKMAN, E. F., AND SCHLABACH, M. R. (1973) *Biochem. Biophys. Res. Commun.* **50,** 84–90.
152. OSUGA, D. T., AND FEENEY, R. E. (1968) *Arch. Biochem. Biophys.* **124,** 560–574.
153. WILLIAMS, J. (1968) *Biochem. J.* **108,** 57–67.
154. WILLIAMS, J. (1962) *Biochem. J.* **83,** 355–364.
155. ELLEMAN, T. C., AND WILLIAMS, J. (1970) *Biochem. J.* **116,** 515–535.
156. GREENE, F. C., AND FEENEY, R. G. (1968) *Biochemistry* **7,** 1366–1371.
157. BEZKOROVAINY, A., GROHLICH, D., AND GERBECK, C. M. (1968) *Biochem. J.* **110,** 765–770.
158. PHILLIPS, J. L., AND AZARI, P. (1971) *Biochemistry* **10,** 1160–1165.
159. BEZKOROVAINY, A., AND GROHLICH, D. (1974) *Comp. Biochem. Physiol.* **47B,** 787–797.
160. TSAO, D., AZARI, P., AND PHILLIPS, J. L. (1974) *Biochemistry* **13,** 397–403.
161. TSAO, D., AZARI, P., AND PHILLIPS, J. L. (1974) *Biochemistry* **13,** 403–408.
162. TSAO, D., AZARI, P., AND PHILLIPS, J. L. (1974) *Biochemistry* **13,** 408–413.
163. WISHNIA, A., WEBER, I., AND WARNER, R. C. (1961) J. Amer. Chem. Soc. **83,** 2071–2080.
164. AZARI, P., AND FEENEY, R. E. (1961) *Arch. Biochem. Biophys.* **92,** 44–53.
165. KOMATSU, S. K., AND FEENEY, R. E. (1967) *Biochemistry* **6,** 1136–1141.
166. LINE, W. F., GROHLICH, D., AND BEZKOROVAINY, A. (1967) *Biochemistry* **6,** 3393–3402.
167. BEZKOROVAINY, A., AND GROHLICH, D. (1971) *Biochem. J.* **123,** 125–126.
168. AISEN, P., AASA, R., MALMSTROM, B. G., AND VANNGARD, T. (1967) *J. Biol. Chem.* **242,** 2484–2490.
169. WOODWORTH, R. C., VIRKAITIS, L. M., WOODBURY, R. G., AND FAVA, R. A. (1975) *in* Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crighton, R. R., ed.), pp. 39–50, North-Holland, Amsterdam.
170. PHELPS, C. F., AND ANTONINI, E. (1975) *Biochem. J.* **147,** 385–391.
171. BATES, G. W., AND SCHLABACH, M. R. (1975) *in* Proteins of Iron Storage and Transport in Biochemistry and Medicine (Crighton, R. R., ed.), pp. 51–58, North-Holland, Amsterdam.
172. WILLIAMS, S. C., AND WOODWORTH, R. C. (1973) *J. Biol. Chem.* **248,** 5848–5853.
173. SCHLABACH, M. R., AND BATES, G. W. (1975) *J. Biol. Chem.* **250,** 2182–2188.
174. AISEN, P., AND LEIBMAN, A. (1968) *Biochem. Biophys. Res. Commun.* **30,** 407–413.
175. AISEN, P., KOENIG, S. H., SCHILLINGER, W. E., SCHEINBERG, I. H., MANN, K. G., AND FISK, W. (1970) *Nature (London)* **226,** 859–861.
176. DONOVAN, J. W., BEARSLEE, R. A., AND ROSS, K. D. (1976) *Biochem. J.* **153,** 631–639.
177. EMERY, T. (1969) *Biochemistry* **8,** 877–882.

178. FLETCHER, J., AND HUEHNS, E. R. (1967) *Nature (London)* **215,** 584–586.
179. HARRIS, D. C., AND AISEN, P. (1975) *Biochemistry* **14,** 262–268.
180. AZARI, P., AND FEENEY, R. E. (1958) *J. Biol. Chem.* **232,** 293–302.
181. EAKIN, R. E., SNELL, E. E., AND WILLIAMS, R. J. (1940) *J. Biol. Chem.* **136,** 801–802.
182. EAKIN, R. E., SNELL, E. E., AND WILLIAMS, R. J. (1941) *J. Biol. Chem.* **140,** 525–543.
183. PARSONS, H. J., GARDNER, J., AND WALLIKER, C. T. (1940) *J. Nutr.* **19,** 19.
184. HERTZ, R., AND SEBRELL, W. H. (1942) *Science* **96,** 257.
185. JONES, P., AND BRIGGS, M. H. (1962) *Life Sci.* **11,** 621–623.
186. FRAPS, R. M., HERTZ, R., AND SEBRELL, W. H. (1943) *Proc. Soc. Exp. Biol. Med.* **52,** 140–142.
187. HERTZ, R. (1949) *Endocrinology* **45,** 451–454.
188. O'MALLEY, B. W., MCGUIRE, W. L., KOHLER, P. O., AND KORENMAN, S. G. (1969) *Recent Prog. Horm. Res.* **25,** 105–153.
189. WHITE, H. B., DENNISON, B. A., FERA, M. A. D., WHITNEY, C. J., MCGUIRE, J. C., MESLAR, H. W., AND SAMMELWITZ, P. H. (1976) *Biochem. J.* **157,** 395–400.
190. MANDELLA, R. D., MESLAR, H. W., AND WHITE, H. B. (1978) *Biochem. J.* **175,** 629–633.
191. DELANGE, R. J. (1970) *J. Biol. Chem.* **245,** 907–916.
192. GREEN, N. M., AND TOMS, E. J. (1970) *Biochem. J.* **118,** 64–70.
193. DELANGE, R. J., AND HUANG, T-S. (1971) *J. Biol. Chem.* **246,** 698–709.
194. HUANG, T-S., AND DELANGE, R. J. (1971) *J. Biol. Chem.* **246,** 686–697.
195. LAUNER, H. F., AND FRAENKEL-CONRAT, H. (1951) *J. Biol. Chem.* **193,** 125–132.
196. GREEN, N. M. (1963) *Biochem. J.* **89,** 585–591.
197. FRAENKEL-CONRAT, H., SNELL, N. S., AND DUCAY, E. D. (1952) *Arch. Biochem. Biophys.* **39,** 97–107.
198. GREEN, N. M. (1963) *Biochem. J.* **89,** 599–609.
199. GREEN, N. M., AND ROSS, M. E. (1968) *Biochem. J.* **110,** 59–66.
200. GREEN, N. M. (1962) *Biochim. Biophys. Acta* **59,** 244–246.
201. GREEN, N. M. (1975) *Advan. Prot. Chem.* **29,** 85–133.
202. GYÖRGI, P., AND ROSE, C. S. (1943) *Proc. Soc. Exp. Biol. Med.* **53,** 55–57.
203. PAI, C. H., AND LICHSTEIN, H. C. (1964) *Proc. Soc. Exp. Biol. Med.* **116,** 197–200.
204. WEI, R-D., AND WRIGHT, L. D. (1964) *Proc. Soc. Exp. Biol. Med.* **117,** 341–344.
205. FRAENKEL-CONRAT, H., SNELL, N. S., AND DUCAY, E. D. (1952) *Arch. Biochem. Biophys.* **39,** 70–96.
206. GREEN, N. M. (1963) *Biochem. J.* **89,** 609–620.
207. GREEN, N. M. (1964) *Biochem. J.* **90,** 564–568.
208. GREEN, N. M., AND MELAMED, M. D. (1966) *Biochem. J.* **100,** 614–621.
209. GREEN, N. M., AND JOYNSON, M. A. (1970) *Biochem. J.* **118,** 71–72.
210. GREEN, N. M., KONIECZNY, L., TOMS, E. J., AND VALENTINE, R. C. (1971) *Biochem. J.* **125,** 781–791.
211. HERTZ, R. (1943) *Proc. Soc. Exp. Biol. Med.* **52,** 15–17.
212. WRIGHT, L. D., AND SKEGGS, H. R. (1944) *Proc. Soc. Exp. Biol. Med.* **56,** 95–98.
213. LANDY, M., DICKEN, D. M., BICKING, M. M., AND MITCHELL, W. R. (1942) *J. Bacteriol.* **43,** 5–6.
214. GREEN, N. M. (1965) *Biochem. J.* **94,** 230.
215. O'MALLEY, B. W., AND KORENMAN, S. G. (1967) *Life Sci.* **6,** 1953–1969.
216. KULOMAA, M., ELO, H. A., AND TUOHIMAA, P. J. (1978) *Biochem. J.* **175,** 685–690.
217. RHODES, M. B., AZARI, P. R., AND FEENEY, R. E. (1958) *J. Biol. Chem.* **230,** 399–409.
218. RHODES, M. B., BENNETT, N., AND FEENEY, R. E. (1959) *J. Biol. Chem.* **234,** 2054–2060.
219. WELLNER, D. (1967) *Annu. Rev. Biochem.* **36,** 669–690.
220. WINTER, W. P., BUSS, E. G., CLAGETT, C. O., AND BOUCHER, R. V. (1967) *Comp. Biochem. Physiol.* **22,** 889–896.
221. OSTROWSKI, W., SKARZYNSKI, B., AND ZAK, Z. (1962) *Biochim. Biophys. Acta* **59,** 517–519.
222. OSTROWSKI, W., ZAK, Z., AND KRAWCZYK, A. (1968) *Acta Biochim. Pol.* **15,** 243–260.
223. FARRELL, H. M., MALLETTE, M. F., BUSS, E. G., AND CLAGETT, C. O. (1969) *Biochim. Biophys. Acta* **194,** 433–442.
224. FARRELL, H. M., BUSS, E. G., AND CLAGETT, C. O. (1970) *Int. J. Biochem.* **1,** 168.
225. CLAGETT, C. O. (1971) *Fed. Proc.* **30,** 127–129.
226. COTNER, R. C., AND CLAGETT, C. O. (1972) *Fed. Proc.* **31,** 851–858.
227. HEIMAN, V. (1935) *Poult. Sci.* **14,** 137–147.
228. NORRIS, L. C., AND BAUERNFIEND, J. C. (1940) *Food Res.* **5,** 521–532.

229. FEENEY, R. E., MEANS, G. E., AND BIGLER, J. C. (1969) *J. Biol. Chem.* **244,** 1957–1960.
230. SNELL, E. E., AND STRONG, F. M. (1939) *Ind. Eng. Chem.* **11,** 346–350.
231. SILBER, R. H., AND MUSHETT, C. W. (1942) *J. Biol. Chem.* **146,** 271–272.
232. BALLS, A. K., AND SWENSON, T. L. (1934) *J. Biol. Chem.* **106,** 409–419.
233. MEYER, K., PALMER, J. W., THOMPSON, R., AND KHORAZO, D. (1936) *J. Biol. Chem.* **113,** 479–486.
234. LINEWEAVER, H., AND MURRAY, C. W. (1947) *J. Biol. Chem.* **171,** 565–581.
235. MATSUSHIMA, K. (1958) *Sciences (N.Y.)* **127,** 1178–1179.
236. RHODES, M. B., BENNETT, N., AND FEENEY, R. E. (1960) *J. Biol. Chem.* **235,** 1686–1693.
237. HANCOCK, R. E. W., HANTKE, K., AND BRAUN, V. (1977) *Arch. Microbiol.* **114,** 231–239.
238. OSUGA, D. T., BIGLER, J. C., UY, L. R., SJOBERG, L., AND FEENEY, R. E. (1974) *Comp. Biochem. Physiol.* **48B,** 519–533.
239. MONSIGNY, M., ADAM-CHOSSON, A., AND MONTREUIL, J. (1968) *Bull. Soc. Chim. Biol.* **50,** 854–886.
240. FEENEY, R. E., ANDERSON, J. S., AZARI, P. R., BENNETT, N., AND RHODES, M. B. (1960) *J. Biol. Chem.* **235,** 2307–2311.
241. NAKAMURA, S., NAGAO, M., AND SUZUNO, R. (1966) *Comp. Biochem. Physiol.* **18,** 937–950.
242. KONINSKY, J. (1979) *in* Bacterial Outer Membranes: Biogenesis and Function (Inouye, M., ed.), pp. 319–359, Wiley-Interscience, New York.
243. VITHAYATHIL, A. J., BUCK, F., BIER, M., AND NORD, F. F. (1961) *Arch. Biochem. Biophys.* **92,** 532–540.
244. BRAUN, V. (1978) *in* 28th Symposium on General Microbiology (Relations between Structure and Function in the Prokaryotic Cell) (Stanier, R. V., Rogers, H. J., and Ward, J. B., eds.), pp. 111–138, Cambridge Univ. Press, London/New York.
245. STEVENS, F. C., AND FEENEY, R. E. (1963) *Biochemistry* **2,** 1346–1352.
246. LIU, W-H., FEINSTEIN, G., OSUGA, D. T., HAYNES, R., AND FEENEY, R. E. (1968) *Biochemistry* **7,** 2886–2892.
247. OZAWA, K., AND LASKOWSKI, M. (1966) *J. Biol. Chem.* **241,** 3955–3961.
248. VINCENT, J-P., AND LAZDUNSKI, M. (1972) *Biochemistry* **11,** 2967–2977.
249. LASKOWSKI, M., AND SEALOCK, R. W. (1971) *in* The Enzymes (Boyer, P., ed.), Vol. 3, pp. 375–473, Academic Press, New York.
250. RYAN, D. S., AND FEENEY, R. E. (1975) *J. Biol. Chem.* **250,** 843–847.
251. FEENEY, R. E. (1971) *in* Proceedings International Research Conference on Proteinase Inhibitors (Fritz, H., and Tschesche, H., eds.), pp. 162–168, de Gruyter, Berlin.
252. MEANS, G. E., RYAN, D. S., AND FEENEY, R. E. (1974) *Acc. Chem. Res.* **7,** 315–320.
253. TOMIMATSU, Y., CLARY, J. J., AND BARTULOVICH, J. J. (1966) *Arch. Biochem. Biophys.* **115,** 536–544.
254. DAVIS, J. G., ZAHNLEY, J. C., AND DONOVAN, J. W. (1969) *Biochemistry* **8,** 2044–2053.
255. RYAN, C. A., CLARK, J. J., AND TOMIMATSU, Y. (1965) *Arch. Biochem. Biophys.* **110,** 175–183.
256. LIU, W-H., MEANS, G. E., AND FEENEY, R. E. (1971) *Biochim. Biophys. Acta* **299,** 176–185.
257. FOSSUM, K., AND WHITAKER, J. R. (1968) *Arch. Biochem. Biophys.* **125,** 367–375.
258. HAINES, R. B. (1938) *J. Hyg.* **37,** 453–461.
259. BOARD, R. G., AYRES, J. C., KRAFT, A. A., AND FORSYTHE, R. R. (1964) *Poult. Sci.* **43,** 584–594.
260. FLORIAN, M. L. E., AND TRUSSELL, P. C. (1957) *Food Technol.* **11,** 56.
261. BOARD, R. G. (1965) *J. Appl. Bacteriol.* **28,** 437–453.
262. BOARD, P. A., AND BOARD, R. G. (1968) *Brit. Poult. Sci.* **9,** 111–120.
263. SEVIOUR, E. M., SYKES, F. R., AND BOARD, R. G. (1972) *Brit. Poult. Sci.* **13,** 549–556.
264. ROSENBERG, H., AND YOUNG, I. G. (1974) *in* Microbial Iron Transport—A Comprehensive Treatise (Neilands, J. B., ed.), pp. 67–82, Academic Press, New York/London.
265. RODGERS, G. C., AND NEILANDS, J. B. (1973) *in* Handbook of Microbiology (Laskin, A. I., and Lechevalier, H. A., eds.), Vol. II, pp. 823–830, CRC Press. Ohio.
266. BIGLAND, C. H., AND PAPAS, G. (1953) *Canad. J. Comp. Med.* **17,** 105–107.
267. FROMM, D., AND MONROE, R. J. (1960) *Food Technol.* **14,** 401–403.
268. RIZK, S. S., AYRES, J. C., AND KRAFT, A. A. (1966) *Poult. Sci.* **45,** 825–829.
269. BÜCHLI, K. (1967) Institut voor de Pluimveeteelt 'Het spelderholt' Beekbergen, Medeling No. 145.
270. VADEHRA, D. V., BAKER, R. C., AND NAYLOR, N. B. (1970) *J. Food Sci.* **35,** 61–62.
271. BOARD, R. G., AND HORNSEY, D. J. (1978) *in* Chemical Zoology (Florkin, M., and Scheer, B. J., eds.), Vol. 10., pp. 37–74, Academic Press, London.

272. FLETCHER, J. (1971) *Immunology* **20,** 493–500.
273. CHANDLEE, G. C., AND FUKUI, G. M. (1965) *Bacteriol. Proc.* **45,** M33.
274. SWORD, C. P. (1966) *J. Bacteriol.* **92,** 536–542.
275. GLADSTONE, G. P., AND WALTON, E. (1971) *Brit. J. Exp. Pathol.* **52,** 452–464.
276. GLADSTONE, G. P., WALTON, E., AND KAY, U. (1974) *Brit. J. Exp. Pathol.* **55,** 427–447.
277. SPIRO, T. G., AND SALTMAN, P. (1969) *Struct. Bonding* **6,** 115–159.
278. WEINBERG, E. D. (1971) *J. Infect. Dis.* **124,** 401–410.
279. BULLEN, J. J., AND ROGERS, H. J. (1969) *Nature (London)* **224,** 380–382.
280. BULLEN, J. J., WILSON, A. B., CUSHNIE, G. H., AND ROGERS, H. J. (1968) *Immunology* **14,** 889–898.
281. FLETCHER, J., AND HUEHNS, E. R. (1968) *Nature (London)* **218,** 1211–1214.
282. ROGERS, H. J. (1973) *Infect. Immun.* **7,** 445–456.
283. GARIBALDI, J. A. (1970) *Appl. Microbiol.* **20,** 558–560.
284. O'BRIEN, I. G., COX, G. B., AND GIBSON, F. (1970) *Biochim. Biophys. Acta* **201,** 453–460.
285. HANCOCK, R. E. W., HANTKE, K., AND BRAUN, V. (1977) *Arch. Microbiol.* **114,** 231–239.
286. GARIBALDI, J. A. (1971) *J. Bacteriol.* **105,** 1036–1038.
287. GARIBALDI, J. A. (1972) *J. Bacteriol.* **110,** 262–265.
288. KOCHAN, I. (1977) *Advan. Chem. Ser.* **162.**
289. HOWEY, P. W., BOARD, R. G., AND KEAR, J. (1977) *Biotelemetry* **4,** 169–180.
290. THEODORE, T. S., AND SCHADE, A. L. (1965) *J. Gen. Microbiol.* **39,** 75–83.
291. THEODORE, T. S., AND SCHADE, A. L. (1965) *J. Gen. Microbiol.* **40,** 385–396.
292. SEVIOUR, E., AND BOARD, R. G. (1972) *Brit. Poult. Sci.* **13,** 557–575.
293. BOARD, R. G., AND HALLS, N. A. (1973) *Brit. Poult. Sci.* **14,** 359–371.
294. KOCHAN, I. (1973) *Curr. Top. Microbiol. Immunol.* **60,** 1–30.
295. ALDERTON, G., AND FEVOLD, H. L. (1946) *J. Biol. Chem.* **164,** 1–6.
296. MANDELES, S. (1960) *J. Chromatogr.* **3,** 256–264.
297. ARNHEIM, N., PRAGER, E. M., AND WILSON, A. C. (1969) *J. Biol. Chem.* **244,** 2085–2094.
298. OSBORNE, T. B., AND CAMPBELL, G. F. (1900) *J. Amer. Chem. Soc.* **22,** 422–450.
299. BAIN, J. A., AND DEUTSCH, H. F. (1948) *J. Biol. Chem.* **172,** 547–548.
300. AZARI, P., AND BAUGH, R. (1967) *Arch. Biochem. Biophys.* **118,** 138–144.
301. MELAMED, M. D., AND GREEN, N. M. (1963) *Biochem. J.* **89,** 591–599.
302. CUATRECAS, P., AND WILCHEK, M. (1968) *Biochem. Biophys. Res. Commun.* **33,** 234–239.

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BACTERIAL GROWTH IN ALBUMEN TAKEN FROM THE EGGS OF DOMESTIC HENS AND WATERFOWL

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SYNOPSIS

Gram-negative bacteria formed small populations in albumen taken from the eggs of domestic hens. Adding yeast extract (final concentration 100 mg/ml) to the albumen did not increase the extent of microbial growth. Large populations were formed in albumen containing $FeSO_4.7H_2O$ (final concentration 9·95 mg/ml) or this sulphate and yeast extract and the organisms utilised the glucose of the egg white with a concomitant increase in the CO_2 released to the atmosphere. The rate of glucose utilisation, as judged by the rate and extent of the change in pH, was slow in albumen of the eggs of domestic hens to which had been added a heavy suspension of a fermentative bacterium. The retardation of glucose fermentation was a feature also of the albumen of eggs of several species of waterfowl. Both the rate and extent of glucose fermentation was increased when yeast extract (final concentration 100 mg/ml) was added to heavily seeded albumen but ferrous sulphate did not have a significant influence on the rate of glucose utilisation.

INTRODUCTION

The inimical properties of albumen taken from the eggs of *Gallus domesticus* were frequently noted (for references see Haines, 1939) in the 25 years following the first systematic study (Gayon, 1873) of the microbiology of the hen's egg. Subsequent studies of purified proteins from the albumen have indicated that it contains several potentially bactericidal or bacteriostatic agents (Board, 1966) but only some of these have as yet been shown or considered to function *in ovo* (Board, 1968).

There has been a tendency to account for the defence of the egg solely in terms of the action of lysozyme—an enzyme of the albumen which lyses certain bacteria (Laschtschenko, 1909; Fleming, 1922). Sharp and Whitaker (1927), however, demonstrated that the alkaline (pH 9·6) reaction of the albumen was important in preventing microbial growth in albumen *in vitro*. Ovotransferrin (conalbumin) was identified by Alderton, Ward and Fevold (1946) as the principal component of the hen's egg's defence. They showed it to be a chelating agent which, through sequestering Fe^{3+}, had inhibited microbial growth in the studies of Schade and Caroline (1944). From this information together with that derived from the many studies of the course of microbial infection of eggs which had been intended for human consumption (Brooks and Taylor, 1955), Board (1969) defined the egg as an ecosystem, the infection of which was impeded by the shell and its underlying membranes

and the colonisation of which was hindered by the antimicrobial properties of the albumen. It was deduced that the latter was the principal component of the hen's egg defence.

Although the defence provided by albumen has itself been the subject of many studies (*e.g.* Rettger and Sperry, 1912; Sharp and Whitaker, 1927; Garibaldi, 1960), the literature does not provide a concise picture of the course of bacterial growth in the albumen *in vitro*. Moreover, it does not provide any clues as to the fate of glucose, the most probable energy source for microbial growth. The present study was undertaken with the objects of establishing the pattern of bacterial growth in and the fate of the glucose of the albumen.

MATERIALS AND METHODS

Eggs of the domestic hens were obtained from a commercial flock of Thornber 404's. The hens were housed in batteries and fed on a proprietary layers' ration. The eggs were kept at room temperature for short-term storage but at 4 °C for storage of more than 1 week. The eggs of waterfowl were obtained from the Wildfowl Trust, Slimbridge. Glos. Their age was not known.

Albumen

The shells of eggs were swabbed with 70% (v/v) ethanol and cracked with a flamed scalpel. The contents were collected in a sterile Petri dish and the white harvested with a 10 ml, wide-bore sterile pipette. The albumen of several eggs was collected in a sterile screw-capped bottle and mixed by gentle shaking. Appropriate volumes (20 ml) of the blended albumen were dispensed in sterile Erlenmeyer flasks (150 ml capacity; closed with a cotton wool bung), or sterile 50 ml beakers (closed with aluminium foil).

The bacteria

The bacteria used in the main part of the work (*Pseudomonas fluorescens*, *Pseudomonas maltophilia*, *Pseudomonas aeruginosa* and *Escherichia coli* C20) had been isolated from rotten eggs (Board and Board, 1968; Seviour, Sykes and Board, 1972). For comparative purposes, bacteria derived from the Department's stock culture collection were used. All the cultures were stored on slopes of Nutrient Agar (Oxoid Ltd) at 4 °C. The bacteria were grown: (*a*) in bottles filled almost to the top with Nutrient Broth (Oxoid) containing 0·5% (w/v) glucose; (*b*) on Nutrient Agar (Oxoid) adhering to one of the large faces of 16 oz " medical flats " having a rectangular cross-section, or (*c*) in vigorously shaken Erlenmeyer flasks containing mineral salts, an ammonium salt and an energy source such as succinate. The actual derivation of the cells used for an inoculum is given in the text. The cells were harvested by centrifugation, washed in sterile distilled water and finally suspended in 5 ml of 0·1 M-phosphate buffer (pH 6·8–7·0). Sterile pipettes were used to inoculate the albumen.

Additives

Analar grade $FeSO_4.7H_2O$ and powdered Yeast Extract (YE) (Oxoid Ltd) were dissolved in glass distilled water and sterilised by autoclaving (1·05 kg/cm^2 for 20 min).

Viable counts

Serial decimal dilutions were prepared by the addition of 1 ml cell suspension to 9 ml quarter-strength Ringer's solution. Known volumes (0·02 ml) of appropriate dilutions were dispensed by the method of Miles and Misra (1938) on to the surface of Nutrient Agar (Oxoid Ltd) the surface of which had been dried by storage at room temperature. The Petri dishes containing the Nutrient Agar were incubated at 27 °C for 24 to 48 h and the colonies counted.

pH

A portable meter (Cambridge Instruments Ltd) was used.

Glucose determination

Two grams of albumen were precipitated with perchloric acid (about 0·33 N) and the precipitate harvested by centrifugation. The supernatant was made up to volume (50 ml) in a volumetric flask and its glucose content determined with the Boehringer kit for determining the glucose concentration in blood.

Collection of CO_2

Round bottomed, three-necked flasks (500 ml; Quickfit Ltd) were sterilised by autoclaving (1·05 kg/cm² for 20 min). One hundred millilitres of albumen were placed in the flask and the latter positioned so that the bottom half was submerged in a water bath maintained at 27 °C. A rubber bung in the centre neck carried two glass tubes. One end of the longer tube was attached via a sterile air filter (cotton wool) and a U-tube containing a carbon dioxide absorbent (Carbosorb; B.D.H.) to a source of compressed air and the other end was positioned about 3 cm above the surface of the albumen. The shorter tube in the central bung was connected by a rubber tube to another glass tube the free end of which was drawn to a capillary. The latter tube was inserted through a rubber bung positioned at one end of a Pettenkofer tube which was inclined and which contained 50 ml of $Ba(OH)_2.8H_2O$ (31·5g/l). The location of the tip of the capillary in the barium hydroxide and the inclination of the Pettenkofer tube were adjusted so that gas bubbles retained their identity when moving along the Pettenkofer tube. At 24-h intervals, the barium hydroxide was collected, the Pettenkofer tube rinsed with glass distilled water and the liquid made up to 100 ml in a volumetric flask. This was titrated with 0·1 N HCl using bromocresol purple as indicator and the amount of CO_2 trapped by the barium hydroxide calculated. In all experiments, CO_2-free air sweeping over uninoculated albumen was bubbled through barium hydroxide, the latter was treated as above and the quantity of CO_2 subtracted from that obtained with an inoculated albumen. The angled side necks were closed with ground glass stoppers which were held in position with adhesive tape. Samples of albumen (10 ml) were taken by introducing a wide-bore pipette through one of the angled necks.

RESULTS

The extent of microbial growth

The general trends noted in this study are shown in Figures 1 and 2. A ten-fold reduction in the number of viable organisms in the 3 h following the inoculation of the albumen of the hen's egg was a common feature. In native albumen, the

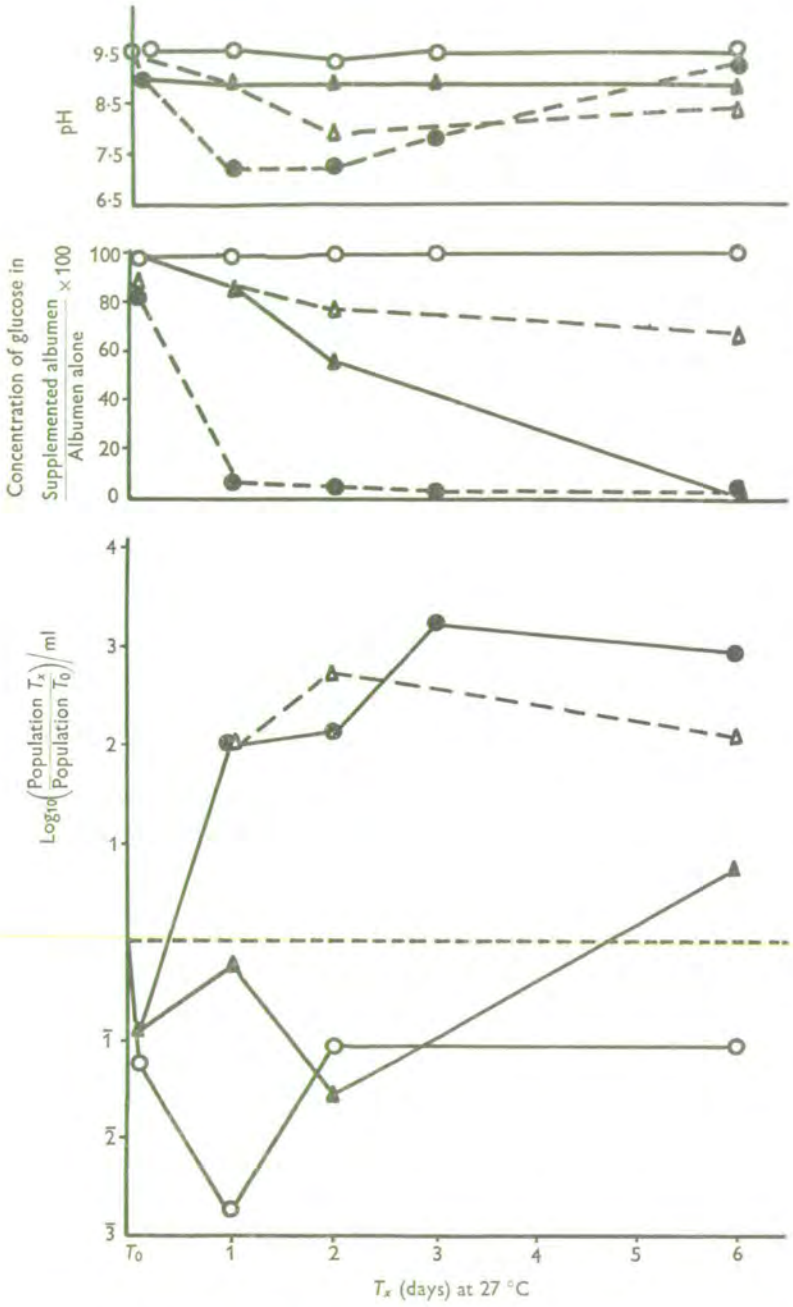

FIG. 1.—The growth of *Escherichia coli* C20 in albumen taken from eggs of domestic hens. The blended albumen (20 ml) of several eggs was held in 150 ml Erlenmeyer flasks at 27 °C and inoculated with a suspension of washed bacterial cells. At the times indicated a flask was randomly selected and changes in the size of the bacterial populations and the glucose and H^+ concentrations in the albumen determined. The results obtained with native albumen (○) was compared with those for albumen containing yeast extract (▲; final concentration 100 mg/ml), Fe^{3+} (△; added as $FeSO_4 . 7H_2O$ in a final concentration of 9·95 mg/ml), and yeast extract and Fe^{3+} in the concentrations noted above (●).

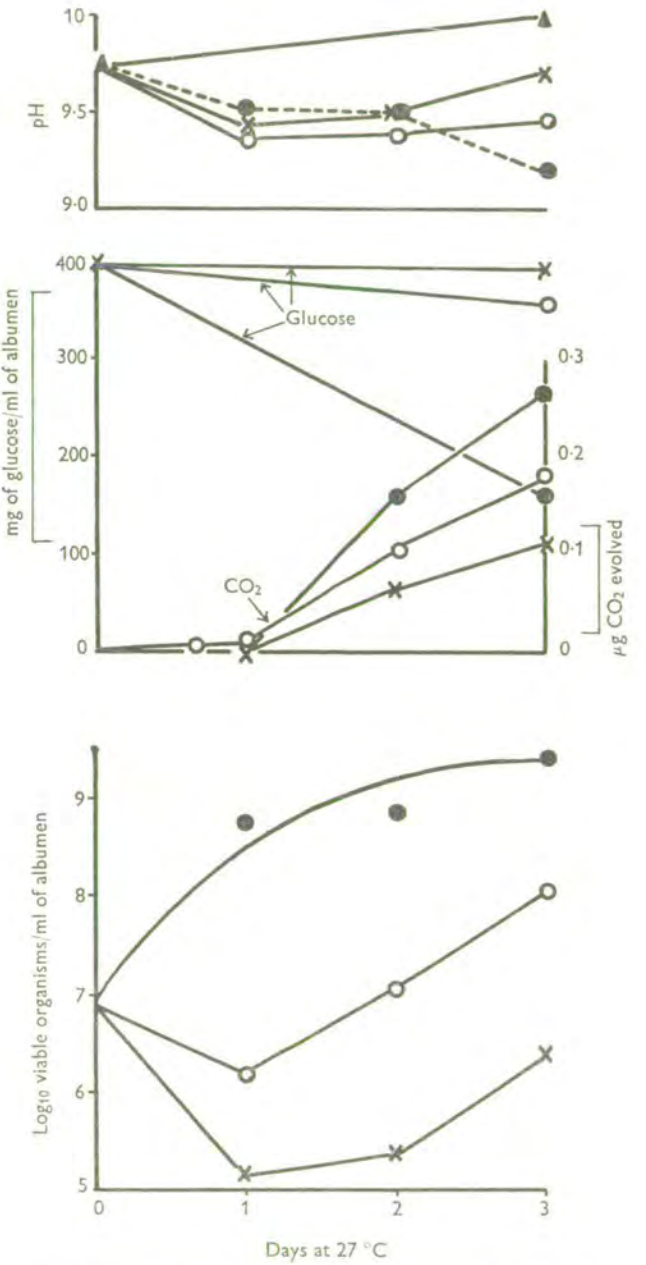

FIG. 2.—The growth of *Pseudomonas fluorescens* in the albumen taken from the eggs of domestic hens. Experimental details are summarised in Figure 1. ▲ = growth in uninoculated albumen; ○ = albumen containing $FeSO_4 \cdot 7H_2O$ (9·95 mg/ml); ● = albumen containing $FeSO_4 \cdot 7H_2O$ (9·95 mg/ml) and yeast extract (100 mg/ml), × = albumen alone.

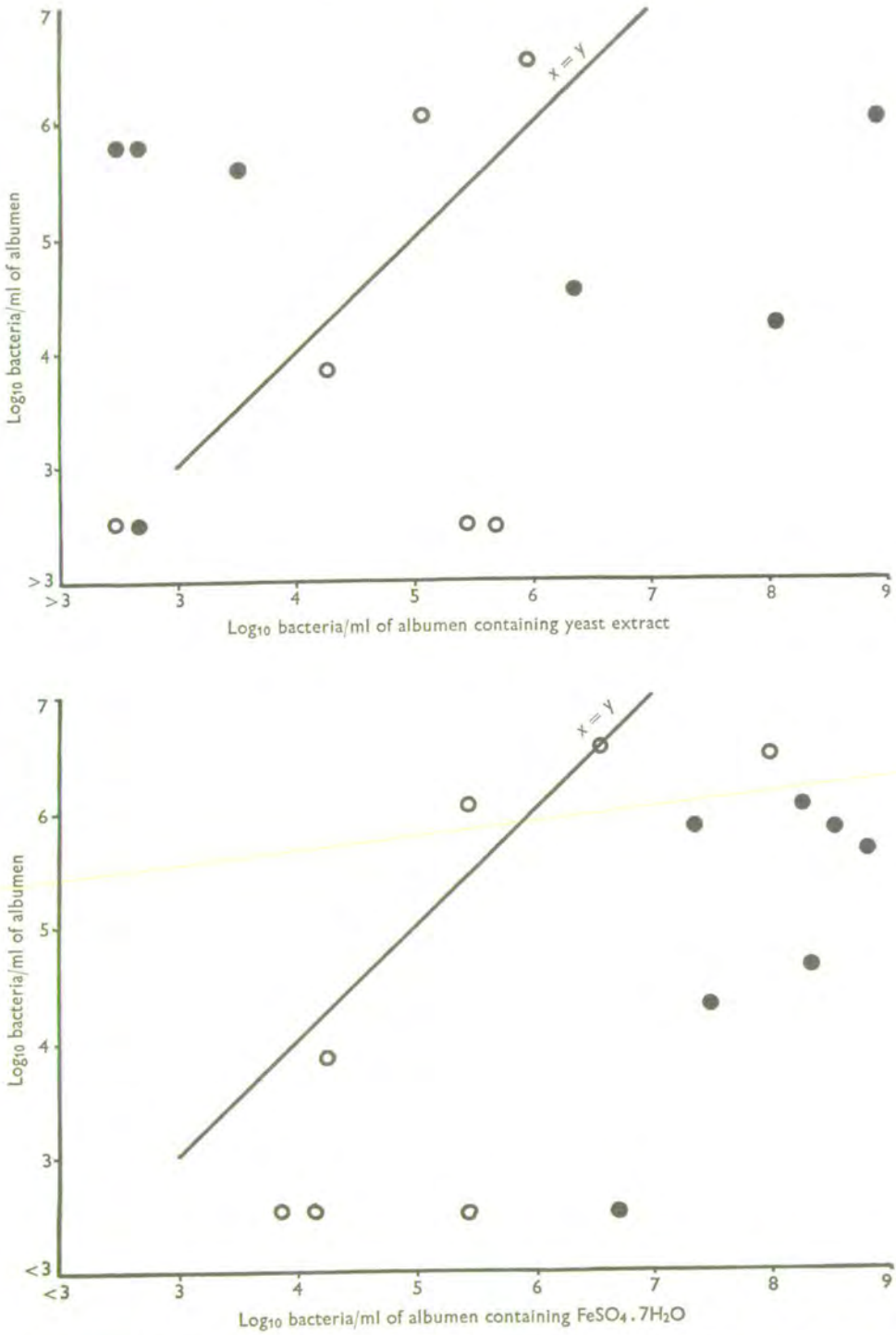

FIG. 3.—The populations achieved by *Escherichia* spp (●) and *Pseudomonas* spp. (○) in albumen taken from the eggs of domestic hens compared with those in albumen supplemented with yeast extract (final concentration 100 mg/ml) or $FeSO_4 . 7H_2O$ (final concentration 9·95 mg/ml). For additional details, see Figure 1.

lag continued for 24 h and, even when microbial growth occurred, the size of the final population was only of the same order as the inocula.

Yeast extract. The addition of YE (final concentration 100 mg/ml) to albumen limited the extent and duration of the lag phase of growth in the experiment recorded in Figure 1, but it resulted in a population which was only slightly larger than that obtaining in native albumen by the 6th d of incubation. It was noted in many experiments that YE protected small inocula in the 24 h following the seeding of albumen. On extended incubation, however, the presence of YE in albumen did not give a consistent pattern in the growth of organisms. In only half of the 13 experiments (Figure 3) were the populations formed in albumen containing YE larger than those in albumen alone.

Iron. When the chelating potential of the ovotransferrin was saturated with Fe^{3+}, extensive bacterial multiplication occurred (Figure 1) and in 12 out of 14 experiments (Figure 3) the populations in albumen containing additional Fe^{3+} were larger than those formed in native albumen.

Yeast extract and iron. The addition of YE (final concentration 100 mg/ml) and Fe^{3+} sufficient to saturate the sequestering potential of ovotransferrin resulted in the extensive multiplication of *E. coli* (Figure 1) and *Ps. fluorescens* (Figure 2). Moreover, the populations achieved by *E. coli* were greater than that in albumen containing additional Fe^{3+} alone (Figure 1). This situation was noted in 13 out of 14 experiments (Figure 4). The growth of coliforms in albumen supplemented with YE and Fe^{3+} resulted in more viable organisms per ml than in a 0·1% (w/v) solution of YE—*e.g.* the concentration at which YE was present in supplemented albumen (Figure 5). In contrast, strains of *Ps. fluorescens* formed the largest populations in YE broth (Figure 5). *Ps. maltophilia* and *Ps. aeruginosa* formed populations of the same magnitude in both media.

These data indicate that the albumen of the hen's egg provides a poor medium for microbial growth and that it is not markedly improved by the addition of YE, a material which provides the organisms with combined nitrogen for synthetic purposes as well as energy substrates. The enhancement of growth by the addition of Fe^{3+} to albumen supports the claim (Schade and Caroline, 1944; Garibaldi, 1960) that ovotransferrin is the major component of the antimicrobial defence of the albumen. Nevertheless, the present study has demonstrated that the saturation of ovotransferrin with Fe^{3+} does not convert albumen to a completely satisfactory medium for microbial growth. Thus the addition of YE to albumen containing additional Fe^{3+} increased the size of the population achieved by the organisms used in this study. Although the addition of YE or Fe^{3+} and YE to albumen led to the extensive growth of bacteria, the rate of multiplication was notably less than that occurring in YE alone. It was found (Figure 6) that, in the majority of experiments, the capacity populations in YE broth were achieved 1 to 2 d following inoculation whereas 6 d were required for supplemented albumen. This evidence suggests that the supplements were neutralising only the major bacteriostatic agents of the albumen.

Glucose and hydrogen ion concentrations and CO_2 evolution

There was no demonstrable change in the glucose or pH of native albumen inoculated with *E. coli* (Figure 1). Likewise, *Ps. fluorescens* (Figure 2) did not reduce

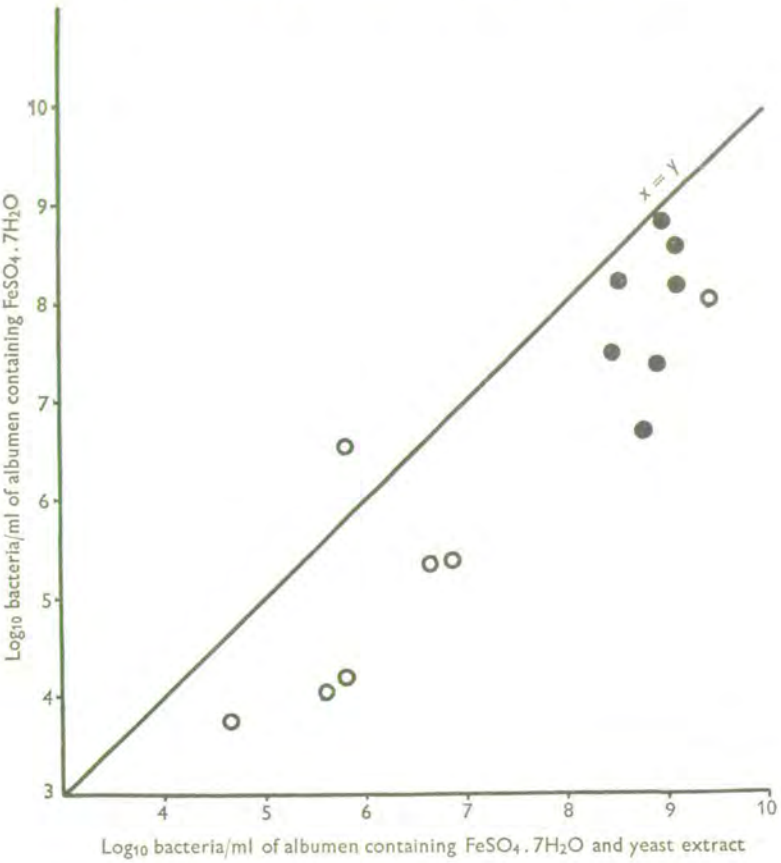

FIG. 4.—The populations achieved by *Escherichia* spp (●) and *Pseudomonas* spp (○) in albumen taken from the eggs of domestic hens and supplemented with $FeSO_4.7H_2O$ (final concentration 9·95 mg/ml) compared with those in albumen containing $FeSO_4.7H_2O$ (final concentration 9·95 mg/ml) and yeast extract (final concentration 100 mg/ml). For additional details, see Figure 1.

the glucose content of unsupplemented albumen although it did cause a slight but temporary acid shift in its reaction. The presence of YE in albumen inoculated with the coliform was associated with a 30% loss of glucose by the 6th d of incubation and a permanent shift of half a pH unit (Figure 1). When iron was the only supplement, the coliform (Figure 1) depleted the albumen of all its glucose whereas the pseudomonad (Figure 2) used only a small part. In both cases, the utilisation of glucose was associated with a drift in the pH, but towards the end of incubation, the pH was reverting to a value similar to that obtaining at the start of the experiment. The addition of Fe^{3+} and YE to albumen resulted in the rapid dissimilation of glucose by the coliform organisms (Figure 1) and the pseudomonads (Figure 2). With the coliforms, the utilisation of glucose was associated with a pronounced fall in the pH (from 9·5 to 7·0). With the pseudomonads (Figure 2), there was a progressive increase in pH in the 3 d of observation.

The total amount of CO_2 recovered from the cultures was correlated with the amount of glucose used (Figures 2 and 7). Thus the largest amounts of CO_2 were

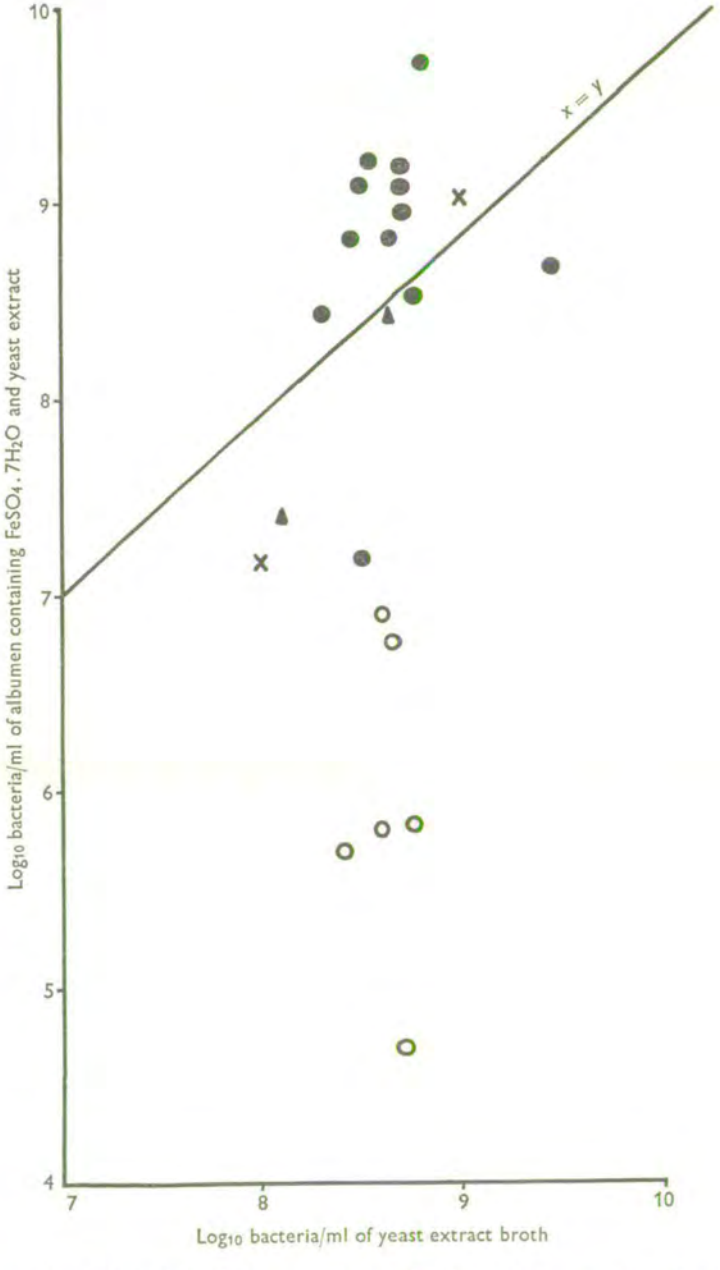

FIG. 5.—The populations achieved by *Escherichia coli* (●), *Pseudomonas fluorescens* (○), *Pseudomonas maltophilia* (×) and *Pseudomonas aeruginosa* (▲) in albumen obtained from the eggs of domestic hens and supplemented with $FeSO_4.7H_2O$ (final concentration 9·95 mg/ml) and yeast extract (final concentration 100 mg/ml) compared with those in 0·1% (w/v) yeast extract broth. For additional details see Figure 1.

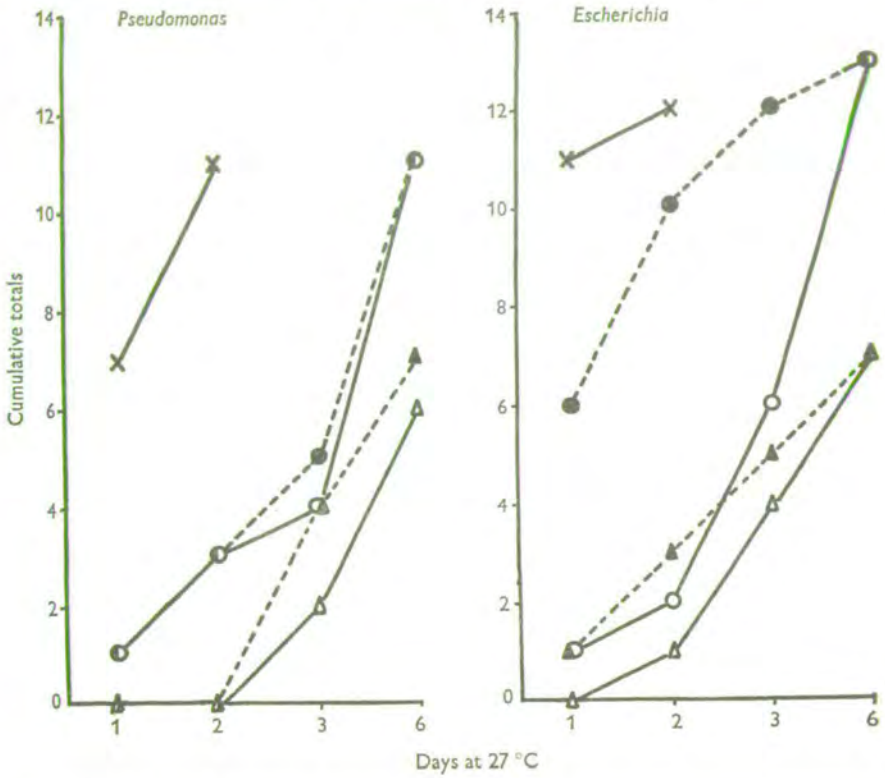

FIG. 6.—The time taken for *Escherichia* spp and *Pseudomonas* spp to achieve climax populations in 0·1% (w/v) yeast extract broth (×), in native albumen (○) taken from the eggs of domestic hens and albumen containing yeast extract (△; final concentration 100 mg/ml), $FeSO_4 \cdot 7H_2O$ (▲; final concentration 9·95 mg/ml) or yeast extract and $FeSO_4 \cdot 7H_2O$ in concentrations noted above (●).

recovered from albumen containing YE and additional Fe^{3+}. There tended, however, to be a lag in the recovery of the CO_2, only small amounts being recovered during the 1 to 2 d following inoculation of the albumen. The major recovery of CO_2 occurred when the pH was reverting to a value similar to that obtaining at the time of inoculation. It is possible that the lag in the recovery of CO_2 was a reflection merely of the impediment offered to gaseous exchange by the viscous albumen. Similarly, the drift in the pH may well have been due to CO_2 influencing the H^+ concentration of the poorly buffered albumen (Figure 9). The growth of coliforms in albumen containing YE and additional Fe^{3+} was always associated with the development of a strong "butter" odour but no success attended attempts to demonstrate diacetyl by the method of Pack, Sandine, Elliker, Day and Lindsay (1964).

This information provides further support for the conclusion that both Fe^{3+} and YE are required to change the albumen of the hen's egg from a medium which supports limited growth of the commonly occurring bacterial contaminants of eggs to one which permits extensive, though retarded (Figure 6), multiplication of these organisms. Moreover, the results obtained from the chemical studies suggest that

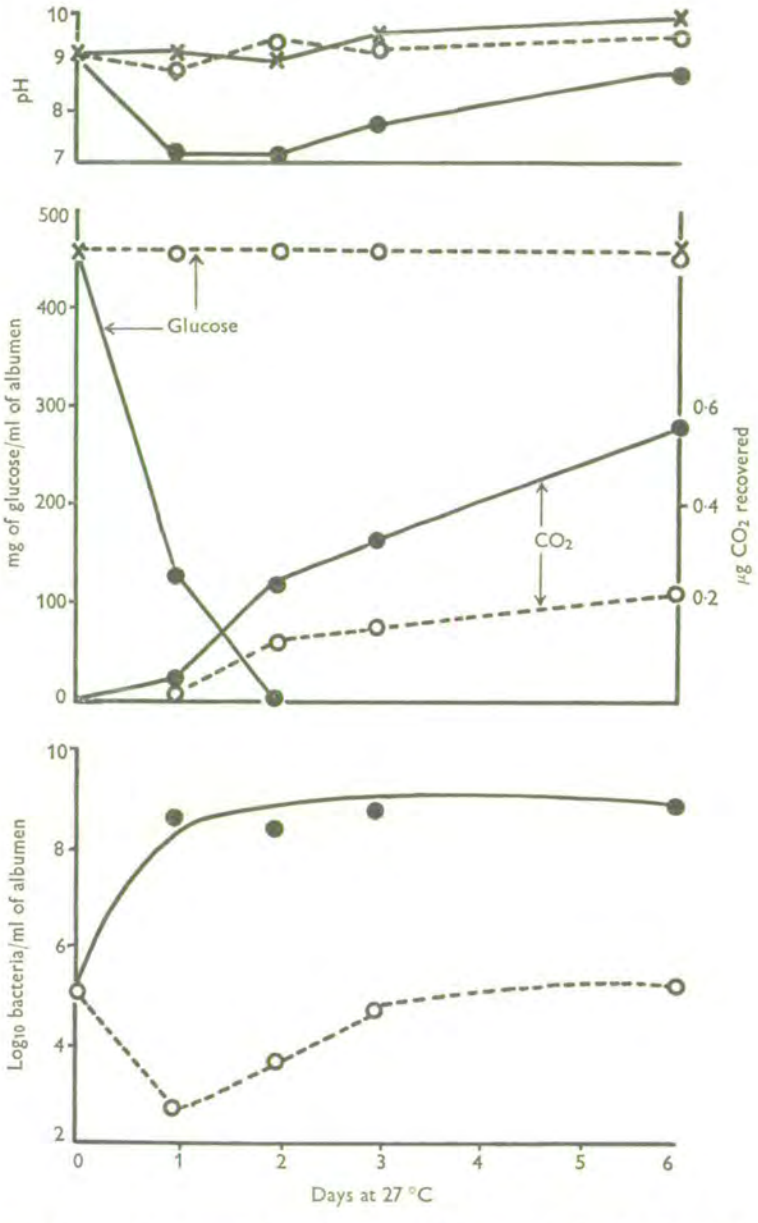

FIG. 7.—Changes in the size of the populations, glucose and H^+ concentrations in albumen derived from the eggs of domestic hens and inoculated with *Escherichia coli* C20. Uninoculated albumen (×), inoculated albumen (○) and albumen supplemented (●) with $FeSO_4 . 7H_2O$ (final concentration 9·95 mg/ml) and yeast extract (final concentration 100 mg/ml).

the antimicrobial defence of the albumen tends to have a " sparing effect " on the glucose contained in the white.

Conservation of glucose

To obtain further information on the " sparing " of glucose, attention was given to the changes in the pH of albumen seeded with a dense suspension of the resting cells of a fermentative bacterium. The pH of hen egg white is about 9·6. Since hen albumen has poor buffering capacity (Cotterill, Gardner, Cunningham

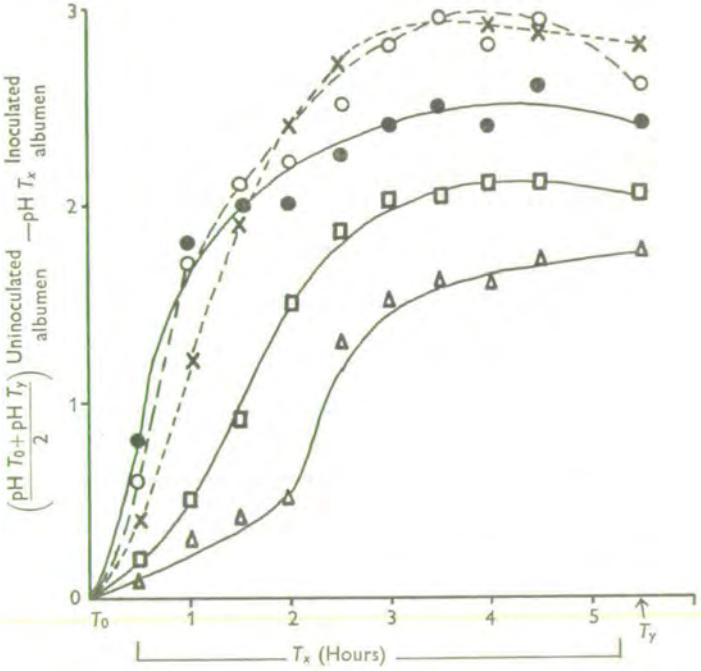

FIG. 8.—The accumulation of H^+ in albumen taken from eggs of domestic hens and seeded with a heavy suspension (equivalent to 60 mg protein* nitrogen/ml albumen) of *Escherichia coli* C20. The albumen from several eggs were blended, seeded with a concentrated suspension of washed cells and 20 ml placed in 50 ml beakers (capped with aluminium foil) to which had been added yeast extract (×; final concentration 100 mg/ml), $FeSO_4 \cdot 7H_2O$ (⊡; final concentration 9·95 mg/ml), or yeast extract and $FeSO_4 \cdot 7H_2O$ (○). Unsupplemented albumen (△) and glucose broth (●) were included also.
* Determined by the method of Minari and Zilversmit (1963).

and Funk, 1958) the acid arising from the fermentation of glucose could be expected to cause the pH to fall towards 6. The results, given in Figures 8 and 9, exemplify those obtained with the albumen of hens' eggs and heavy suspensions of *E. coli* C20. Preliminary experiments emphasised the need for heavy bacterial suspensions if the observations were to be completed in a short period. The changes in pH in native albumen (Figure 8) gave an S-shaped curve over a range of 1·75 units. The addition of sufficient Fe^{3+} to saturate the chelating potential of ovotransferrin enhanced acid production but not to the same extent as did yeast extract alone. The latter was used in a final concentration of 100 mg/ml because it was noted

(Table 1) that this was the concentration which gave the most rapid fall in pH. The addition of Fe^{3+} to a YE-supplemented albumen tended to enhance slightly the rate and extent of the accumulation of H^+. It was noted (Figure 9) that these

TABLE 1

Influence of the concentration of yeast extract on the change of pH in albumen seeded with a heavy suspension (equivalent to 60 mg protein/ml) of Escherichia coli *C20*

Concentration of yeast extract (mg/ml of albumen)	pH change[1]
0	1·35
25	2·25
50	2·25
75	2·65
100	2·9
250	2·95
750	3·1
1000	3·2
1250	3·2
1500	2·75
2000	3·0

[1] Recorded after $4\frac{1}{2}$ h incubation at 27 °C.

TABLE 2

Changes[1] in the glucose and H-ion concentrations in albumen seeded with Escherichia coli[2] *C20*

Albumen taken from eggs of	Held in	Additions	pH change (units)	Percentage glucose utilised
Gallus domesticus	Open beaker	None	1·65	20·5
		$FeSO_4.7H_2O$ (9·95 mg/ml)*	1·95	25·2
		Yeast extract (100 mg/ml)	2·7	100
		$FeSO_4.7H_2O$ and yeast extract (concentrations as above)	2·5	100
	Hypodermic syringe	None	1·6	58·5
		$FeSO_4.7H_2O$ (9·95 mg/ml)*	2·0	40·5
		Yeast extract (100 mg/ml)*	2·8	100
		$FeSO_4.7H_2O$ and yeast extract (concentrations as above)	2·7	100
Mallard	Open beaker	None	1·35	13·5
		$FeSO_4.7H_2O$ (9·95 mg/ml)*	2·05	12
		Yeast extract (100 mg/ml)*	3·4	47·0
		$FeSO_4.7H_2O$ and yeast extract (concentrations as above)	3·4	43·5

[1] Recorded after $5\frac{1}{2}$ h at 27 °C.
[2] Inoculum equivalent to 60 mg protein/ml.
* Final concentrations.

changes in the pH occurred over a range in which the albumen had little buffering capacity. Results similar to those in Figure 8 were obtained in albumen seeded with dense suspensions of the following: *Aeromonas liquefaciens*, *Proteus vulgaris*, *Salmonella*

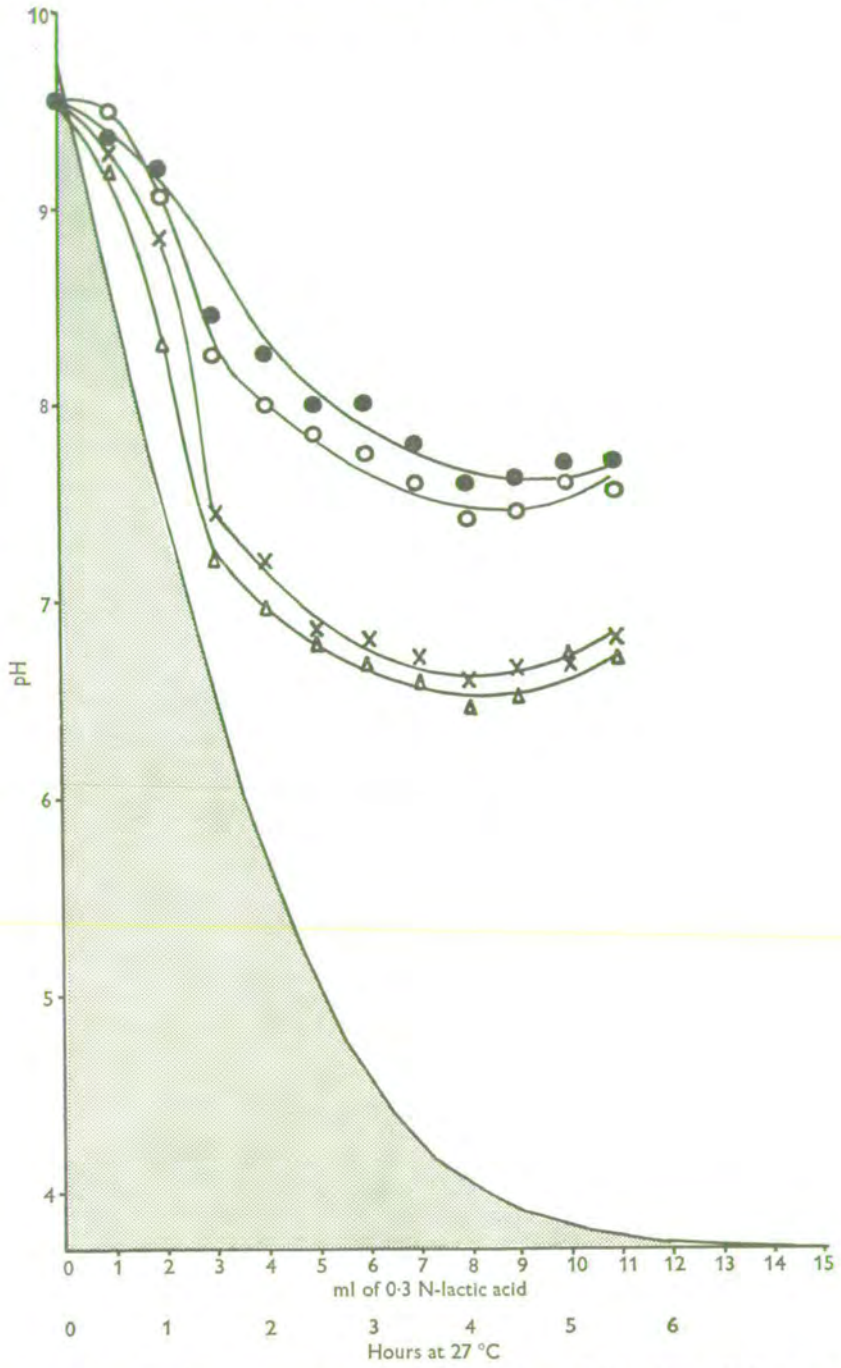

FIG. 9.—The pH drift in albumen containing heavy suspensions of *Escherichia coli* C20 and the titration curve for albumen. ● = unsupplemented albumen; ○ = albumen containing $FeSO_4 \cdot 7H_2O$ (final concentration 9·95 mg/ml); × = albumen containing yeast extract (final concentration 100 mg/ml), and △ = albumen containing $FeSO_4 \cdot 7H_2O$ and yeast extract at concentrations noted above. Stippled area, curve obtained from titration of uninoculated albumen with 0·3N lactic acid.

waycross, *Salmonella brandenberg*, *Serratia marcescens* and other strains of *E. coli*. Moreover, the same general pattern was observed (Figure 10) when dense suspensions of

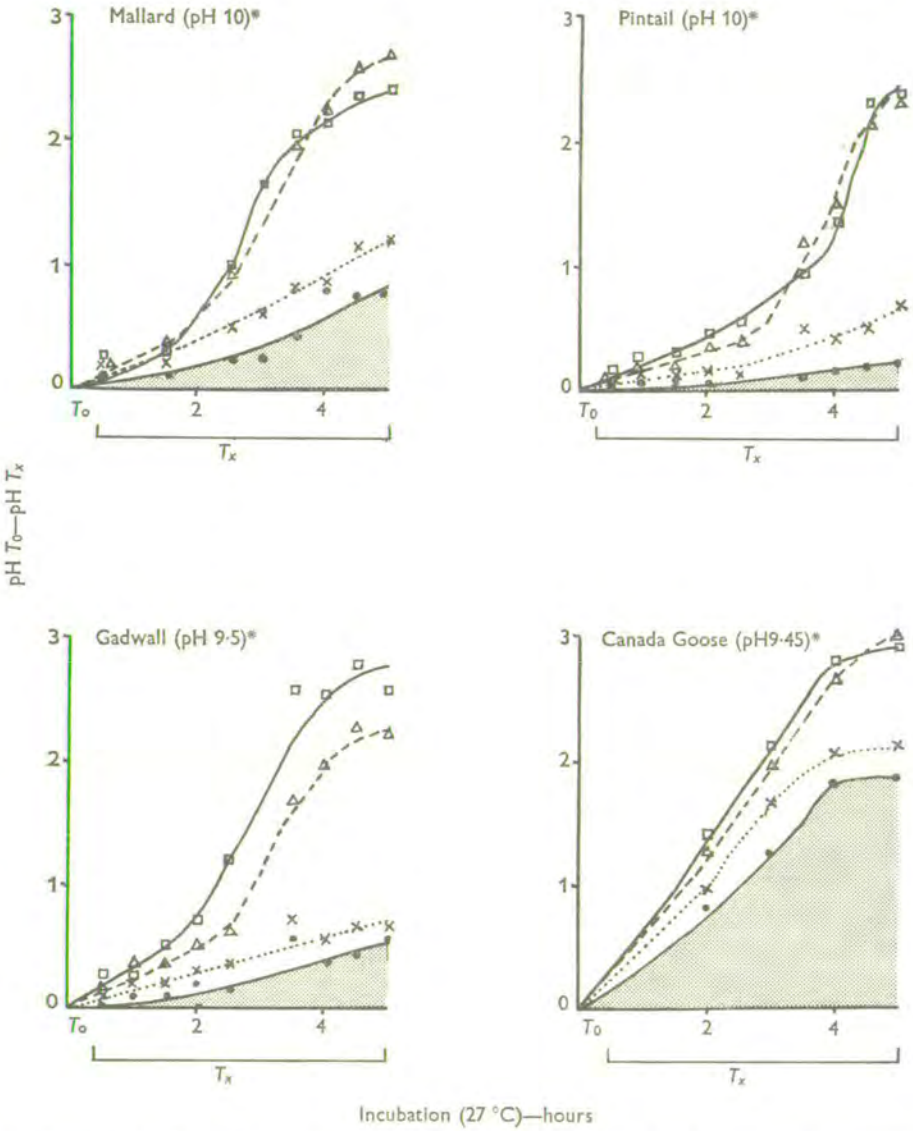

FIG. 10.—Changes in pH caused by heavy suspensions (equivalent to 60 mg protein/ml) of *Escherichia coli* C20 in albumen taken from the eggs of waterfowl. *, initial pH of the albumen; stippled area, native albumen; dotted line, albumen containing $FeSO_4 \cdot 7H_2O$ (final concentration 9·95 mg/ml); broken line, albumen containing yeast extract (final concentration 100 mg/ml), and solid line, albumen containing $FeSO_4 \cdot 7H_2O$ and yeast extract in concentrations noted above.

E. coli C20 were incubated in albumen obtained from the eggs of water fowl. The results given in Table 2 indicate that the change in H-ion concentrations of the albumen reflected changes in the concentration of glucose and that this substrate

was attacked most rapidly when YE was present in albumen. Thus the results given in Figures 9 and 10 show that the albumen of domestic hens and water fowls retard the fermentation of glucose by facultatively anaerobic Gram-negative bacteria.

In the experiments discussed in this section, the resting cells were harvested from bottles filled almost to the top with glucose broth. Thus anaerobic conditions would have obtained shortly following inoculation and the cells would have been adapted to the fermentation of glucose. It was notable, therefore, that results similar to those given in Figure 9 were obtained with cells harvested from vigorously shaken Erlenmeyer flasks, containing a solution of mineral salts, an ammonium salt and succinate, or from the surface of nutrient agar. Similarly, the choice of containers for the inoculated albumen did not appear to be important. Thus the pH recorded at the 5th h of incubation of 20 ml of inoculated albumen (in a 50 ml beaker) which had been vigorously stirred at 30-min intervals with the electrode of a pH meter was the same as that for a 5 ml sample of the same albumen which had been held (in a water bath at 27 °C) in the barrel of a plastic hypodermic syringe (" Plastipak ", Becton, Dickinson, Ireland) the needle boss of which had been sealed with a gentle flame. Similarly, the extent of glucose utilisation (Table 2) was of the same order. Contact between the piston of the hypodermic syringe and the inoculated albumen was not broken during incubation and attempts (a sharp tap, increase in temperature etc.) failed to release gas from the albumen.

DISCUSSION

The results obtained in this study have confirmed earlier reports that the unavailability of iron through chelation by ovotransferrin is the principal impediment to the growth of bacteria in the albumen of the hen's egg (Schade and Caroline, 1944; Brooks 1960; Garibaldi, 1960, 1970). The initial studies of the bacteriostatic activity of ovotransferrin showed that the presence of another chelating agent (Feeney and Nagy, 1952) in a medium containing purified ovotransferrin could overcome bacteriostasis presumably as a result of iron being made available by the competitive inhibition of one chelate by another. It is well known that bacteria and moulds growing in media rendered iron-deficient by precipitation and/or sequestration produce iron transport compounds such as fluorescent hydroxamate, 2,3-dihydroxybenzoylserine, etc. (Garibaldi and Neilands, 1956; Ito and Neilands, 1958; Brot and Goodwin, 1968) and that production can be enhanced by using mutants which have an impaired capacity for the synthesis of iron-containing metabolites (Cox, Gibson, Luke, Newton, O'Brien and Rosenberg, 1970). Moreover, these iron transport compounds need not have a high specificity for a particular organism. Thus *Arthrobacter terregens* can be used to assay (Reich and Hanks, 1964) the mycobactins for which *Mycobacterium paratuberculosis* has an obligate requirement (Snow, 1970). In the light of this evidence it has been suggested that, during their initial suspension in egg white, bacteria would be able to synthesise iron transport compounds which would negate the bacteriostatic action of ovotransferrin and thus lead to large populations (Garibaldi, 1960; Garibaldi and Bayne, 1962*a*). It is noteworthy that large populations are formed by bacteria in egg white supplemented with fluorescent hydroxamate or enterobactin (Garibaldi, 1970) or in serum

containing 2,3-dihydroxybenzoylserine (Wilkins and Lankford, 1970)—the addition of iron to serum *in vitro* removes bacteriostasis due to transferrin (Schade and Caroline, 1946). The evidence obtained in this study would seem to argue against the ability of the commonly occurring contaminants of eggs being able to overcome the bacteriostatic action through the synthesis of iron transport compounds in native egg albumen. Moreover, studies with mixed bacterial infections in the shell membranes *in situ* (Seviour and Board, 1972) did not indicate that the organisms were ameliorating the unfavourable environment within these structures. It has been established that contamination of the site of infection of the shell membrane with traces of iron (Garibaldi and Bayne, 1962*a*) leads to extensive microbial growth and gross contamination of the albumen (Board, Hendon and Board, 1968) and rapid rotting of the eggs (Garibaldi and Bayne, 1960, 1962*b*). A similar response was observed (Board, 1964) when bacteria suspended in extracts of soil or faeces were placed on the shell membranes *in situ*. In the latter study, treatment of the extracts with 8-hydroxyquinoline reduced but did not eliminate the enhancement of bacterial growth in the membranes. This has been noted repeatedly (F. R. Sykes and R. G. Board, unpublished) with inocula on the shell membranes of fertile and infertile eggs held at 38 °C. Thus factors other than iron appear to play a part in promoting bacterial growth during the initial phases of microbial infection of eggs and it may be in this situation that iron transport compounds contained in soil or hen's faeces are important.

Recent reports indicate that the transferrins' rôle in antimicrobial defence systems is not confined to eggs. Thus the injection of iron into experimentally infected animals has been shown to enhance microbial growth and/or reduce the LD_{50} dosage (Jackson and Burrows, 1956; Martin, Jandl and Finland, 1963; Summers and Hasenclever, 1964; Sword, 1966). There is perhaps an unfortunate tendency to consider (*e.g.* Wilkins and Lankford, 1970) that transferrins are components of " non-specific defence mechanisms ". If viewed from the standpoint of classical immunology, then perhaps there is not the stereochemical specificity which is the basis of the antigen-antibody reaction. Nevertheless, transferrins by chelating iron could be expected to have a specific effect on the physiology of those organisms which require this ion. Theodore and Schade (1965) have shown that ovotransferrin in an aerated culture of *Staphylococcus aureus* results in the organism having an enzymic constitution similar to that which it has when growing anaerobically on glucose—the enzymes of glycolysis being abundant but those of respiration sparse (Strasters and Winkler, 1963).

Both Haines (1939) and Brooks (1960) considered that the low level of non-protein nitrogen in the albumen of the hen's egg would hamper microbial growth. The results obtained in the present study indicate that large populations of bacteria are formed in albumen supplemented with Fe^{3+} and that these were only increased slightly when yeast extract was present. Further work is required to establish whether it is the amino acids or the growth factors of yeast extract which are promoting growth under these conditions. It was found that yeast extract leads to the utilisation of glucose by bacteria growing or merely suspended in the albumen. This phenomenon was noted by Ayres (1958), but the rôle of yeast extract in accentuating microbial fermentation of the glucose has yet to be elucidated.

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REFERENCES

ALDERTON, G., WARD, W. H. AND FEVOLD, H. L. (1946). Identification of the bacteria-inhibiting, iron-binding protein of egg white as conalbumin. *Archs Biochem.*, **11**: 9-13.

AYRES, J. C. (1958). Methods for depleting glucose from egg albumen before drying. *Fd Technol. Champaign*, **12**: 186-189.

BOARD, P. A. AND BOARD, R. G. (1968). A diagnostic key for identifying organisms recovered from rotten eggs. *Br. Poult. Sci.*, **9**: 111-120.

BOARD, P. A., HENDON, L. P. AND BOARD, R. G. (1968). The influence of iron on the course of bacterial infection of the hen's egg. *Br. Poult. Sci.*, **9**: 211-215.

BOARD, R. G. (1964). The growth of Gram-negative bacteria in the hen's egg. *J. appl. Bact.*, **27**: 350-364.

BOARD, R. G. (1966). Review article: the course of microbial infection of the hen's egg. *J. appl. Bact.*, **29**: 219-341.

BOARD, R. G. (1968). Microbiology of the egg: a review. In: *Egg Quality: A Study of the Hen's Egg*, pp. 133-162. Edit. CARTER, T. C. Edinburgh, Oliver and Boyd.

BOARD, R. G. (1969). The microbiology of the hen's egg. *Adv. appl. Microbiol.*, **11**: 245-281.

BROOKS, J. (1960). Mechanisms of the multiplication of *Pseudomonas* in the hen's egg. *J. appl. Bact.*, **23**: 459-509.

BROOKS, J. AND TAYLOR, D. I. (1955). Eggs and Egg Products. *Rep. Fd Invest. Bd No.* **60**. London, Her Majesty's Stationery Office.

BROT, N. AND GOODWIN, J. (1968). Regulation of 2,3-dihydroxybenzoylserine synthetase by iron. *J. biol. Chem.*, **243**: 510-513.

COTTERILL, O. J., GARDNER, F. A., CUNNINGHAM, F. E. AND FUNK, E. M. (1958). Titration curves and turbidity of whole egg white. *Poult. Sci.*, **37**: 479-483.

COX, G. B., GIBSON, F., LUKE, R. K. J., NEWTON, N. A., O'BRIEN, I. G. AND ROSENBERG, H. (1970). Mutation affecting iron transport in *Escherichia coli*. *J. Bact.*, **100**: 219-226.

FEENEY, R. B. AND NAGY, D. A. (1952). The antibacterial activity of the egg white protein conalbumin. *J. Bact.*, **64**: 629-643.

FLEMING, A. (1922). On a remarkable bacteriolytic element found in tissues and secretions. *Proc. R. Soc.*, **B93**: 306-317.

GARIBALDI, J. A. (1960). Factors in egg white which control growth of bacteria. *Fd Res.*, **25**: 337-344.

GARIBALDI, J. A. (1970). Role of microbial iron transport compounds in the bacterial spoilage of eggs. *Appl. Microbiol.*, **20**: 558-560.

GARIBALDI, J. A. AND BAYNE, H. G. (1960). The effect of iron on the pseudomonas spoilage of experimentally infected shell eggs. *Poult. Sci.*, **39**: 1517-1520.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*a*). Iron and the bacterial spoilage of shell eggs. *J. Fd Sci.*, **27**: 57-59.

GARIBALDI, J. A. AND BAYNE, H. G. (1962*b*). The effect of iron on the *Pseudomonas* spoilage of farm washed eggs. *Poult. Sci.*, **41**: 850-853.

GARIBALDI, J. A. AND NEILANDS, J. B. (1956). Formation of iron-binding compounds by micro-organisms. *Nature, Lond.*, **177**: 526-527.

GAYON, M. H. (1873). Sur l'alteration spontanée des oeufs. *Compt. Rendus.*, **76**: 232-233.

HAINES, R. B. (1939). Microbiology in the preservation of the hen's egg. *Rep. Fd Invest Bd*, No. **47**. London, Her Majesty's Stationery Office.

ITO, T. AND NEILANDS, J. B. (1958). Products of "low-iron fermentation" with *Bacillus subtilis*:

isolation, characterization and synthesis of 2,3-dihydroxybenzoylglycine. *J. Am. chem. Soc.*, **80**: 4645-4647.

JACKSON, S. AND BURROWS, T. W. (1956). The virulence-enhancing effect of iron on non-pigmented mutants of virulent stains of *Pasteurella pestis*. *Br. J. exp. Path.*, **37**: 577-583.

LASCHTSCHENKO, P. (1909). Uber die keimtotende und entwicklungschemmende Witkung von Auhneseiweiss. *Z. Hyg. InfectKrankh.*, **64**: 419-427.

MARTIN, C. M., JANDL, J. H. AND FINLAND, M. (1963). Enhancement of acute bacterial infections in rats and mice by iron and the inhibition by human transferrin. *J. infect. Dis.*, **112**: 158-163.

MILES, A. A. AND MISRA, S. S. (1938). The estimation of bactericidal power of the blood. *J. Hyg., Camb.*, **38**: 732-745.

MINARI, O. AND ZILVERSMIT, D. B. (1963). Use of KCN for stabilization of colour in direct nesslerization of Kjeldahl digests. *Analyt. Biochem.*, **6**: 320-328.

PACK, M. Y., SANDINE, W. E., ELLIKER, P. R., DAY, E. A. AND LINDSAY, R. C. (1964). Owades and Jackovac method for diacetyl determination in mixed-stain starters. *J. Dairy Sci.*, **47**: 981-986.

REICH, C. V. AND HANKS, J. H. (1964). Use of *Arthrobacter terregens* for bioassay of mycobactin. *J. Bact.*, **87**: 1317-1320.

RETTGER, L. E. AND SPERRY, J. A. (1912). The antiseptic and bactericidal properties of egg-white. *J. med. Res.*, **21**: 55-64.

SCHADE, A. L. AND CAROLINE, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science, N.Y.*, **100**: 14-15.

SCHADE, A. L. AND CAROLINE, L. (1946). An iron-binding component in human blood plasma. *Science, N.Y.*, **104**: 340-341.

SEVIOUR, E. M. AND BOARD, R. G. (1972). The behaviour of mixed bacterial infections in the shell membranes of the hen's egg. *Br. Poult. Sci.*, **13**: 33-43.

SEVIOR, E. M., SYKES, F. R. AND BOARD, R. G. (1972). A microbiological survey of the incubated eggs of chickens and waterfowl. *Br. Poult. Sci.*, **13**: 549-556.

SHARP, P. R. AND WHITAKER, R. (1927). The relation of the hydrogen ion concentration of egg white to its germicidal action. *J. Bact.*, **14**: 17-46.

SNOW, G. A. (1970). Mycobactins: iron chelating growth factors from mycobacteria. *Bact. Rev.*, **34**: 99-125.

STRASTERS, K. C. AND WINKLER, K. C. (1963). Carbohydrate metabolism in *Staphylococcus aureus*. *J. gen. Microbiol.*, **33**: 213-231.

SUMMERS, D. F. AND HASENCLEVER, H. F. (1964). *In vitro* inhibition of yeast growth by mouse ascites fluid and serum. *J. Bact.*, **87**: 1-7.

SWORD, C. P. (1966). Mechanisms of pathogenesis in *Listeria monocytogenes* infection. 1. Influence of iron. *J. Bact.*, **92**: 536-542.

THEODORE, T. S. AND SCHADE, A. L. (1965). Carbohydrate metabolism of iron-rich and iron-poor *Staphylococcus aureus*. *J. gen. Microbiol.*, **40**: 385-395.

WILKINS, T. D. AND LANKFORD, C. E. (1970). Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine, and its stimulation of growth in human serum. *J. infect. Dis.*, **121**: 129-136.

Br. Poult. Sci., 14: 359-371. 1973 *Longman: printed in Great Britain*

THE EFFECT OF IRON ON THE GROWTH OF *ESCHERICHIA COLI* IN ALBUMEN TAKEN FROM THE HEN'S EGG

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SYNOPSIS

Small inocula of *Escherichia coli* did not initiate either growth or glucose utilisation in hen's egg white *in vitro* unless ovotransferrin was quenched with iron. The most rapid and extensive growth occurred in iron-supplemented albumen containing additional ammonium ions. In albumen containing a large inoculum, glucose utilisation was promoted by the addition of NH_4^+ but not Fe^{3+}. The action of NH_4^+ was suppressed by the addition of chloramphenicol to heavily seeded albumen.

INTRODUCTION

In discussions of avian embryology there is a tendency to ignore those systems which, through protecting the white and yolk from microbial infection, permit embryo development in an environment isolated from that of the parents. Tokin (1964) has emphasised the need for an antimicrobial defence and others (*e.g.* Girfanova, 1949; Korotkova, 1957; Mouchan, 1962) have shown that the white of the hen's egg is inimical to bacteria, moulds, spermatozoa, etc. They appear to have gone no further than showing that toxicity is not due to lysozyme alone. Following the observation (Schade and Caroline, 1944) that the bacteriostatic action of albumen is due principally to the chelation of iron by ovotransferrin (Alderton *et al.*, 1946; Feeney and Nagy, 1952), it has been established that this ligand is the main component in the defence against rotting of commercial eggs by Gram-negative bacteria (Garibaldi, 1960; Seviour and Board, 1972). The behaviour of Gram-negative bacteria in albumen appears to be analogous to that of related organisms in fresh vertebrate serum wherein transferrins behave as bacteriostats (Fletcher, 1971). The present study sought to identify further factors which impede the growth of *Escherichia coli* and, especially, its utilisation of glucose in albumen held at the body temperature of the hen.

MATERIALS AND METHODS

Albumen

The eggs were obtained from a commercial flock of brown-egg layers housed in batteries and fed on a proprietory layers' ration. The shells were cracked and

the contents collected in a sterile Petri dish. A wide-bore 10-ml pipette was used to harvest the albumen and that from several eggs was mixed in a sterile Erlenmeyer flask. Twenty-five or 30 ml of blended albumen were placed in a 100-ml Erlenmeyer flask and, following the addition of supplements and an inoculum, kept in a water bath at the temperature noted in the text.

Bacteria

The majority of the organisms were recovered from rotten eggs (Seviour *et al.*, 1972), the others were taken from the Department's collection of stock cultures. They were maintained on slopes of Nutrient Agar (Oxoid) at 4 °C. Inocula were prepared from 18 h Nutrient Agar or Nutrient Broth (Oxoid) cultures. The cells were harvested in sterile distilled water and washed twice in water.

Viable counts

The method of Miles and Misra (1938) was used.

Glucose determinations

A sample (1 ml) of albumen was precipitated in 10 ml 0·33N perchloric acid and the glucose content determined with the Boehringer blood sugar kit.

Anaerobic culture

Albumen was held for 48 h in an anaerobic jar containing a cold catalyst and H_2 (95 parts) : CO_2 (5 parts). It was inoculated with a washed suspension of test organism and incubated in anaerobic jars.

Serum

Pooled human serum obtained from a medical laboratory was filter-sterilised and used in the same manner as egg white.

RESULTS

The extent of growth, as determined by the number of generations occurring in 24 h, and the amount of glucose utilised were used in a survey to assess the efficacy (Fig. 1) of supplements in modifying albumen so that it would permit the initiation of growth by a small inoculum of the coliform organism. There was little or no growth or glucose utilisation when the organism was suspended in albumen alone or albumen supplemented with substances such as casamino acids which contained nothing other than extraneous contamination with Fe^{3+}. When the chelating potential of ovotransferrin was quenched with this metal, the coliform grew (2 to 6 generations/24 h) and there was about 50% reduction in the albumen's content of glucose. The addition of casamino acids, growth factors and trace elements to albumen containing Fe^{3+} resulted in growth (13 generations/24 h) and extensive utilisation of glucose. Growth of this magnitude was obtained, however, simply by adding ammonium ions to iron supplemented albumen—the actual iron salt did not appear to influence the result (Fig. 1). During the first 4 d of incubation, there is a rapid transfer of water from the albumen to the sub-blastodermic fluid (New, 1956).

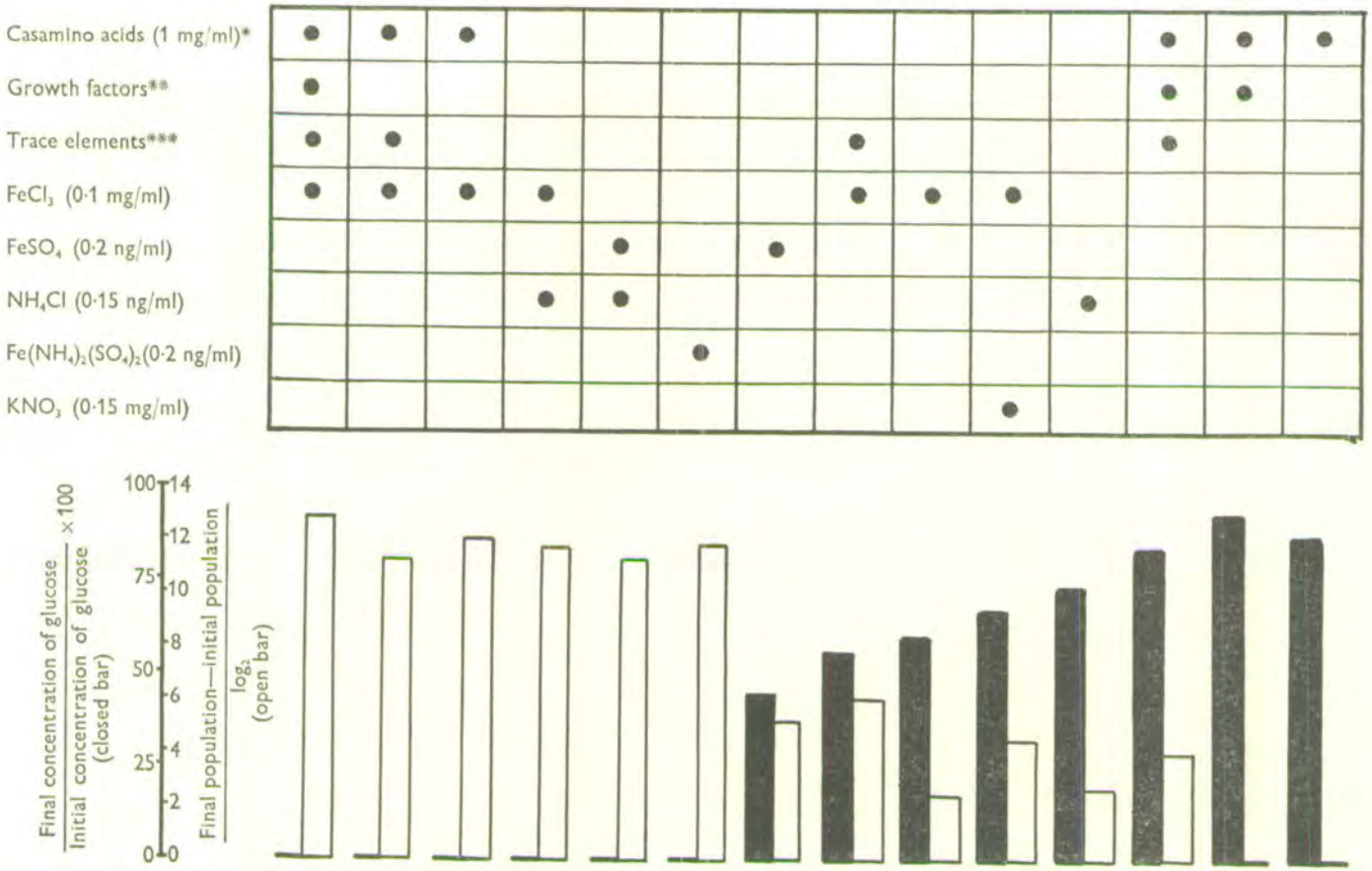

FIG. 1.—The effect of supplements on the growth of *Escherichia coli* in albumen held at 39·5 °C. *, final concentration in albumen; ** (final concentration in parenthesis), *para*-aminobenzoic acid (10 mg/ml); folic acid (1 mg/ml); cyanocobalamin (1 mg/ml); nicotinic acid (1 mg/ml); pantothenic acid (1 mg/ml) thiamine (1 mg/ml), riboflavin (1 mg/ml), and biotin (1 mg/ml), and *** (final concentration in parenthesis), K_2HPO_4 (0·5 mg/ml); NaCl (0·1 mg/ml); $MnSO_4$; $4H_2O$ (2·0 mg/ml); $Na_2MoO.2H_2O$ (2·0 mg/ml); $ZnSO_4$ (0·1mg/ml); $MgSO_4$ $7H_2O$ (0·2 mg/ml), and $CaCl_2$ (0·05 mg/ml).

It would appear from the results given in Table 1 that the water activity of the albumen is not lowered to such an extent that it accentuates the inimical properties of the albumen.

TABLE 1

Effect of drying on growth of Escherichia coli *in albumen*

	No. viable organisms/ml			mg glucose/ml		
Time (days)	0	1	2	0	1	2
Control albumen	$4{\cdot}2 \times 10^3$	$3{\cdot}5 \times 10^3$	$1{\cdot}6 \times 10^4$	3·08	3·03	3·30
Control albumen + 0·2 mg/ml $Fe(NH_4)_2(SO_4)_2$(FAS)	$5{\cdot}7 \times 10^3$	$2{\cdot}6 \times 10^8$	...	2·70	0	...
Desiccated albumen[1]	$3{\cdot}2 \times 10^3$	$2{\cdot}8 \times 10^3$	$6{\cdot}0 \times 10^3$	4·18	3·52	5·58
Desiccated albumen + 0·2 mg/ml FAS	$4{\cdot}1 \times 10^3$	$3{\cdot}4 \times 10^8$	...	4·02	0	...
Desiccated albumen + H_2O	$1{\cdot}2 \times 10^3$	$1{\cdot}1 \times 10^4$	$1{\cdot}6 \times 10^4$	3·25	3·03	3·25
Desiccated albumen + H_2O + 0·2 mg/ml FAS	$2{\cdot}6 \times 10^3$	$2{\cdot}3 \times 10^8$	...	2·97	0	...

[1] Albumen held for 6 d in desiccator with P_2O_5 until 22·1 ml (%) water lost.

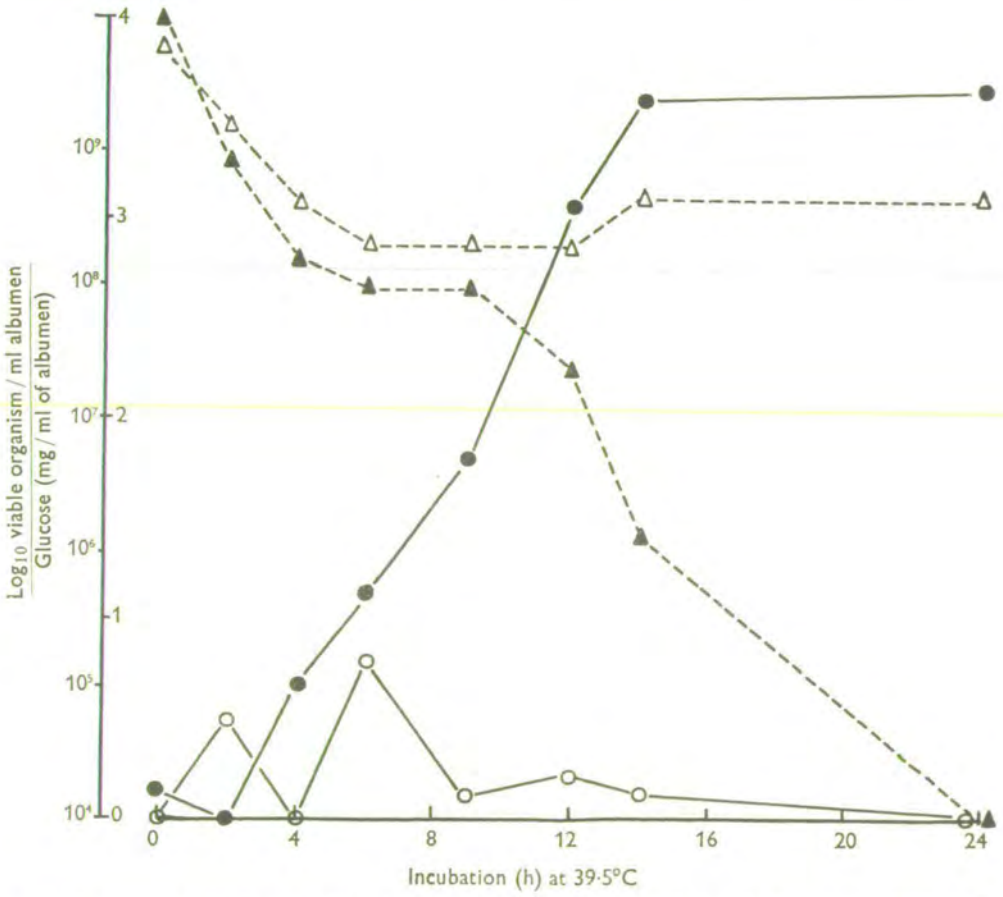

FIG. 2.—The growth (solid lines) of *Escherichia coli* in albumen (open circles) and albumen containing 0·8 μM $Fe(NH_4)_2(SO_4)_2$/ml (closed circles). The broken line gives the concentration of glucose in the control (open triangles) and supplemented (closed triangles) albumen. Temperature of incubation, 39·5 °C.

The growth of *E. coli* in albumen with and without $Fe(NH_4)_2(SO_4)_2$ is shown in Fig. 2. The changes in numbers of viable organisms in supplemented albumen are typical of those occurring in a nutrient broth and similar to those reported for fresh human serum to which Fe^{3+} had been added (Fletcher, 1971). Our isolate multiplied (Fig. 3) in pooled human serum containing $FeCl_3$ or $Fe(NH_4)_2(SO_4)_2$ but not in serum containing ammonium ions. There was a hundredfold decrease in the population of coliforms in unsupplemented serum. The organisms remained viable in albumen (Fig. 2) and the quiescent organisms could be induced to multiply by

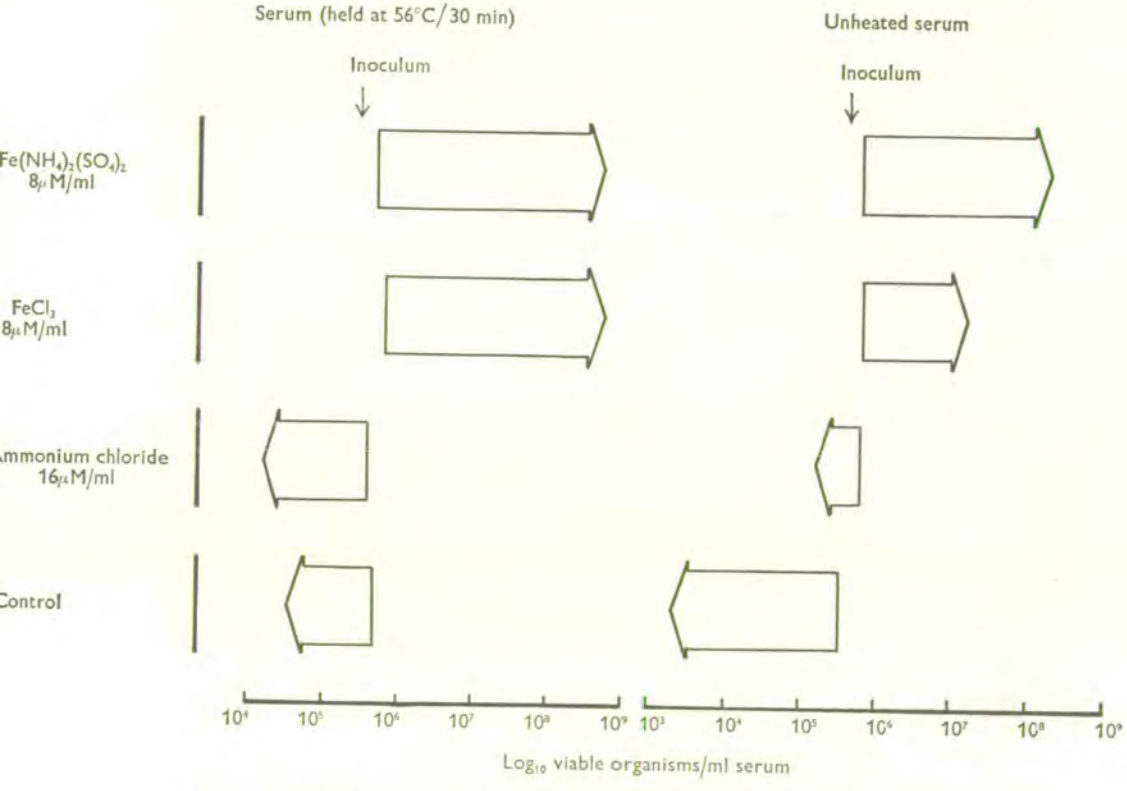

FIG. 3.—Growth of *Escherichia coli* in pooled human serum incubated at 37 °C.

the addition of $Fe(NH_4)_2(SO_4)_2$ to that which had been seeded 3, 4 or 5 d prior to the addition. This suggests that bacteriostasis did not render the coliform sensitive to lysozyme. In a survey (Fig. 4) of isolates typical of those which are present in rotten eggs (Seviour *et al.*, 1972), it was noted that *Bacillus* spp. were rapidly lysed by lysozyme but that *E. coli* and *Pseudomonas* spp. were only lysed when EDTA was present in an alkaline (pH 8·0) solution of lysozyme. The organisms included in this survey were tested for their ability to remain viable in albumen held at 4, 25 and 41 °C. As would be expected from the data (Fig. 4) on lysozyme sensitivity, the *Bacillus* spp. were killed by the albumen at all temperatures of storage (Fig. 5) whereas *Micrococcus* spp. and *Staphylococcus aureus* remained viable in albumen even though they tended to die in phosphate buffer. With the Gram-negative bacteria,

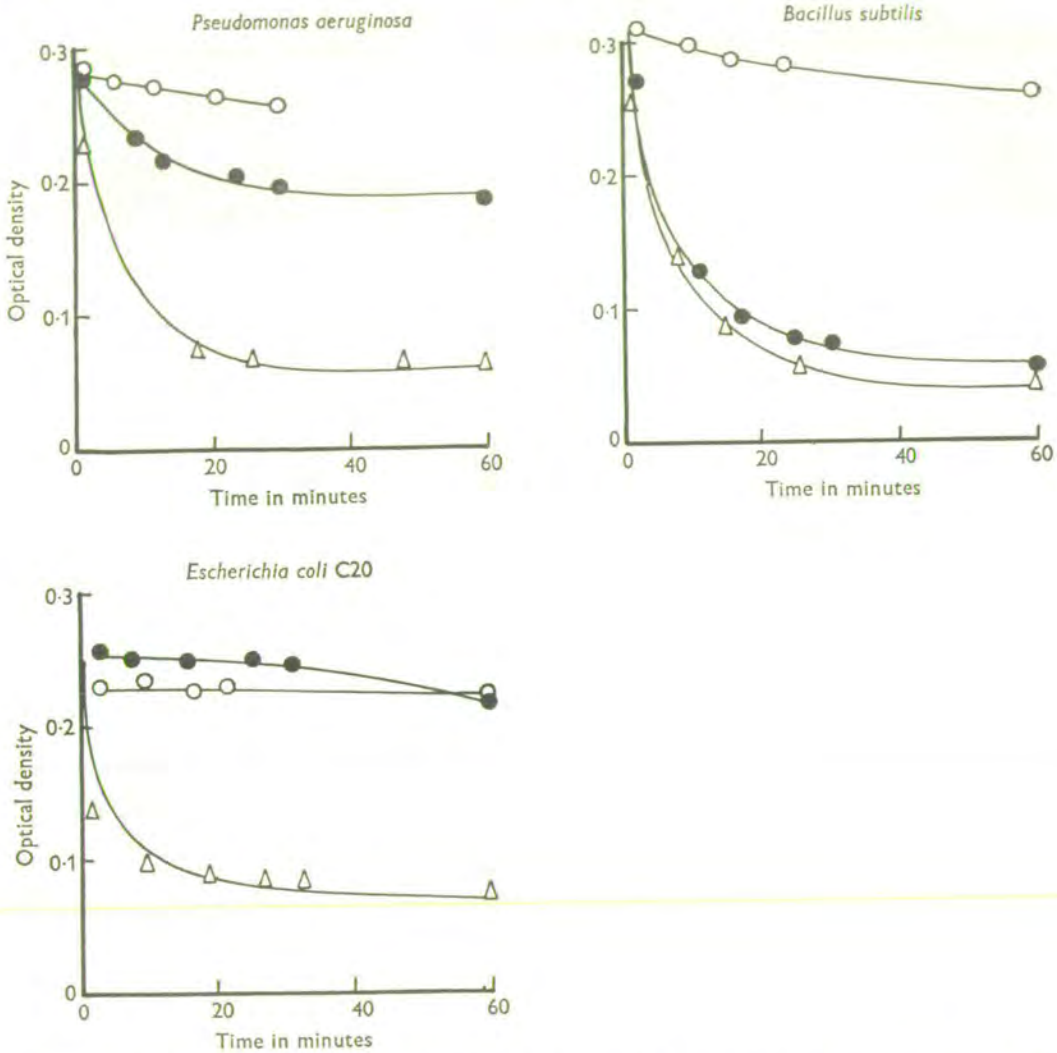

FIG. 4.—The lysis of bacteria. A washed suspension of bacteria was suspended in tris buffer (M/30, pH 8·0) (○); tris buffer containing lysozyme (20 mg/ml) (●) or the latter containing ethylene diamine tetra-acetic acid (500 mg/ml) (△). The suspensions were held at 20 °C and optical density determined at 660 nm.

viability was retained by organisms suspended in albumen at 4 or 25 °C. The mesophilic organisms, with the exception of a strain of coliform, remained viable in albumen at 41 °C (Fig. 6), but, at this temperature, the psychrotrophs (*Ps. fluorescens*, *Ps. maltophilia*, *Ps. putida* and *Acinetobacter*) died. This evidence indicates that iron-deprivation does not make Gram-negative bacteria liable to lysis by lysozyme.

Holding inoculated albumen in an O_2-free atmosphere did not result (Fig. 7) in the growth of *E. coli* unless the albumen had been supplemented with $Fe(NH_4)(SO_4)_2$. This suggests that albumen, in addition to depriving an organism of Fe^{3+}, has a property whereby a facultative anaerobe is prevented from obtaining

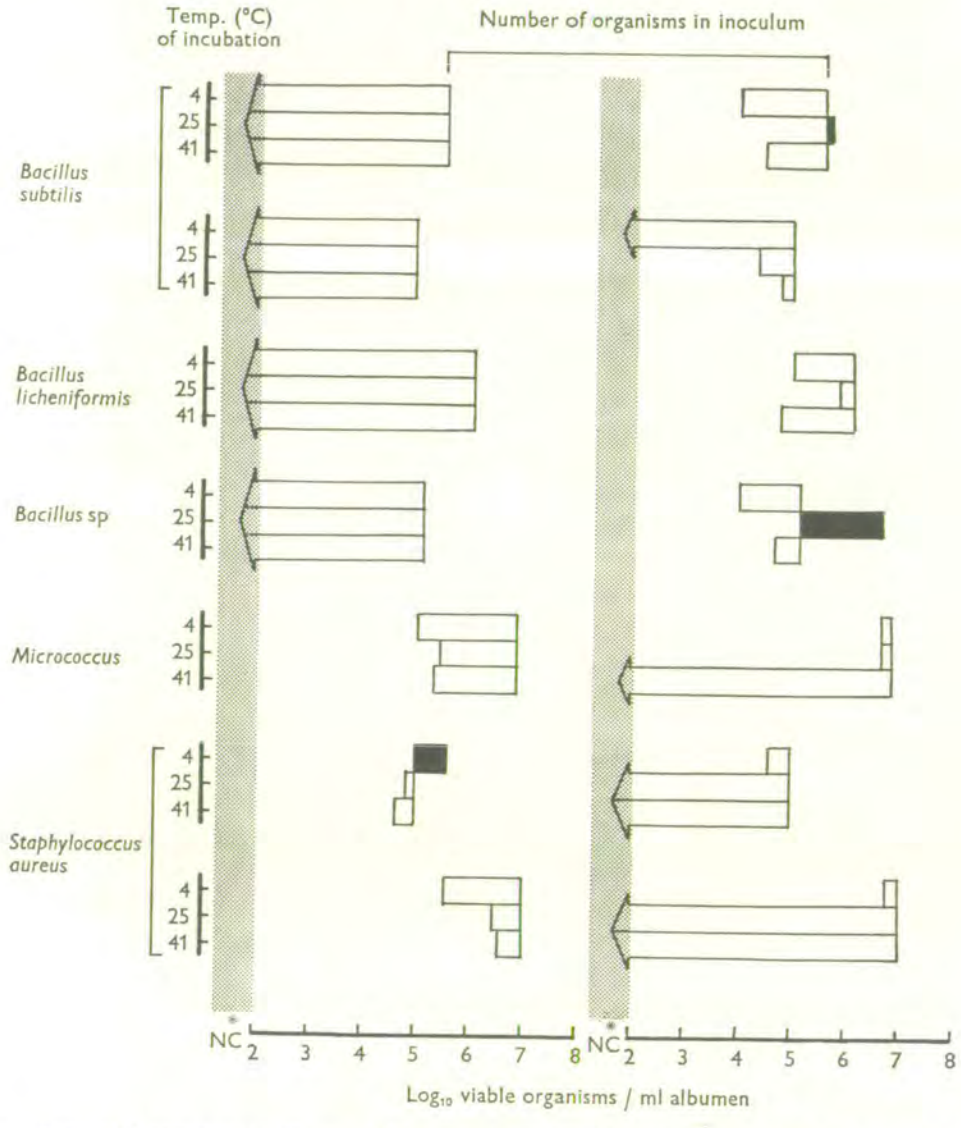

FIG. 5.—Survival of organisms in albumen held at 4, 25 or 41 °C. Open bars, decrease in size of population, and closed bars, increase in size of population. *NC, viable organisms not recovered.

energy through glycolysis. This hindrance of fermentation—as indicated by the rate and extent of H-ion accumulation—was evident in albumen containing a very heavy seeding of *E. coli* (Fig. 8). It was overcome by the addition of ammonium ions but only partially by Fe^{3+}. The enhancement of fermentation by ammonium ions was suppressed by chloramphenicol (Fig. 9) thus indicating that the low content of non-protein nitrogen in albumen provides an impediment to protein synthesis by organisms which are attempting to adapt from respiration to fermentation. This may also be accentuated by alkaline shock of the inoculum. We have noted an

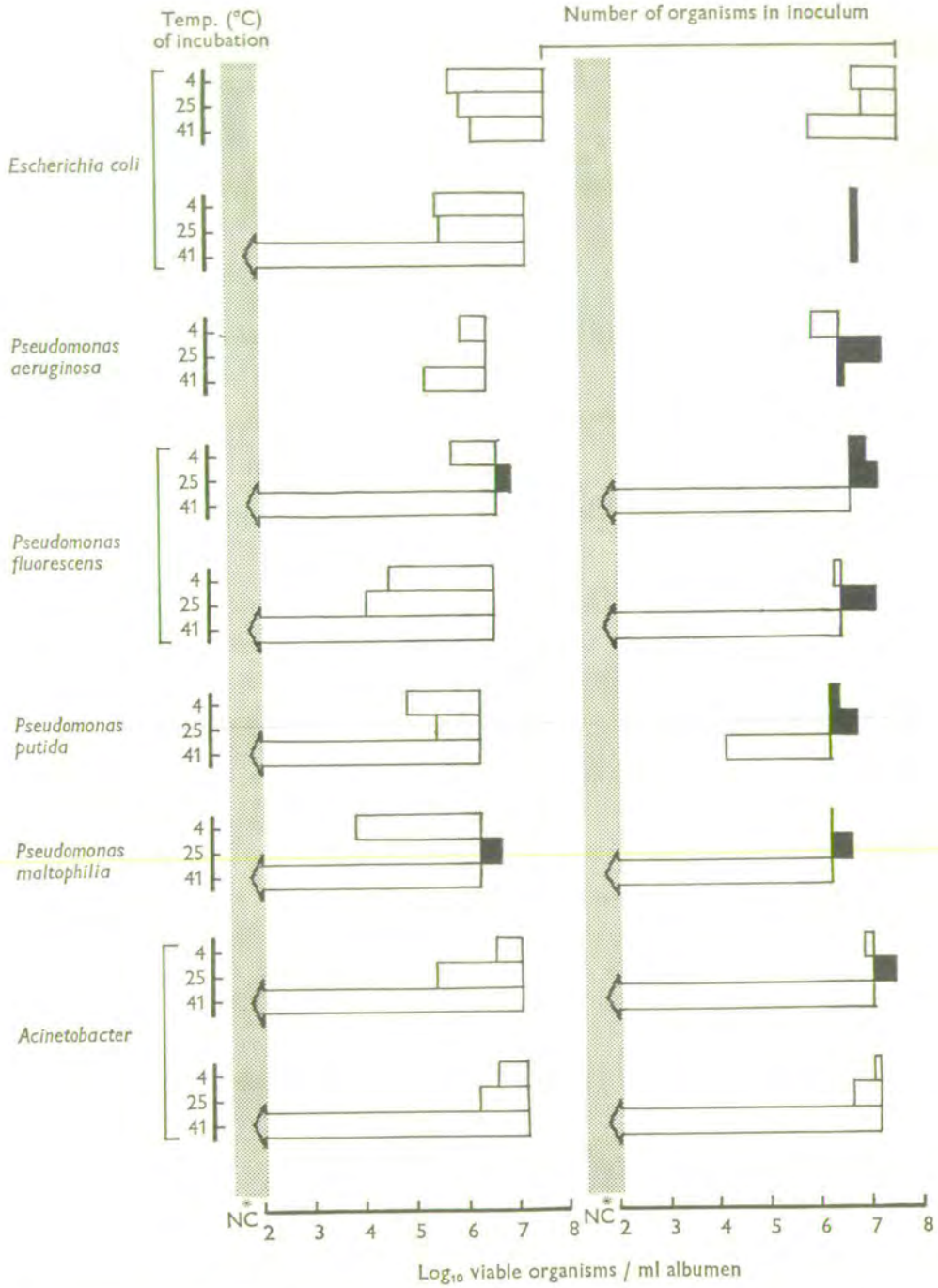

FIG. 6.—Survival of organisms in albumen held at 4, 25 or 41 °C. Open bars, decrease in size of population, and closed bars, increase in size of population. *NC, viable organisms not recovered.

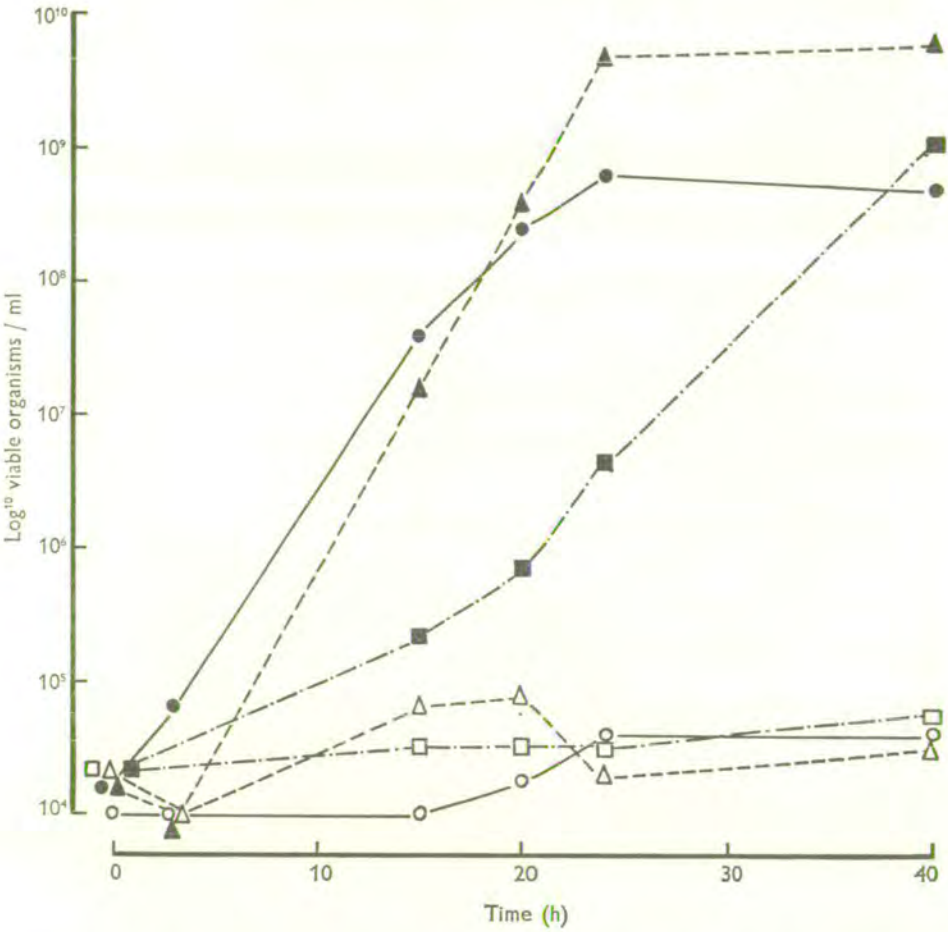

FIG. 7.—The growth of *Escherichia coli* in albumen incubated in an atmosphere of H_2 (95 parts) : CO_2 (5 parts). Closed symbols, albumen containing 2 mg/ml $Fe(NH_4)_2(SO_4)_2$, and open symbols, albumen alone. Incubation was at 15 ° (dot and dash), 25 ° (dash) and 37 °C (solid line).

increase in ninhydrin-positive substances in a bicarbonate buffer (pH 9·6) containing a sachet of dialysis tubing filled with albumen (pH 9·0) and a heavy suspension of *E. coli*. There was only a small accumulation of such substances when the albumen was neutralised before inoculation.

DISCUSSION

This study has shown that the unavailability of Fe^{3+} through chelation by ovotransferrins is the principal impediment to the growth of *E. coli* in albumen held at the hen's body temperature. In this respect, ovotransferrin acts in a manner analogous to that of transferrins in fresh human serum (Schade and Caroline, 1946; Schade, 1963; Fletcher, 1971). There is, however, one important difference between the two systems; iron-deprived *E. coli* and related organisms remain quies-

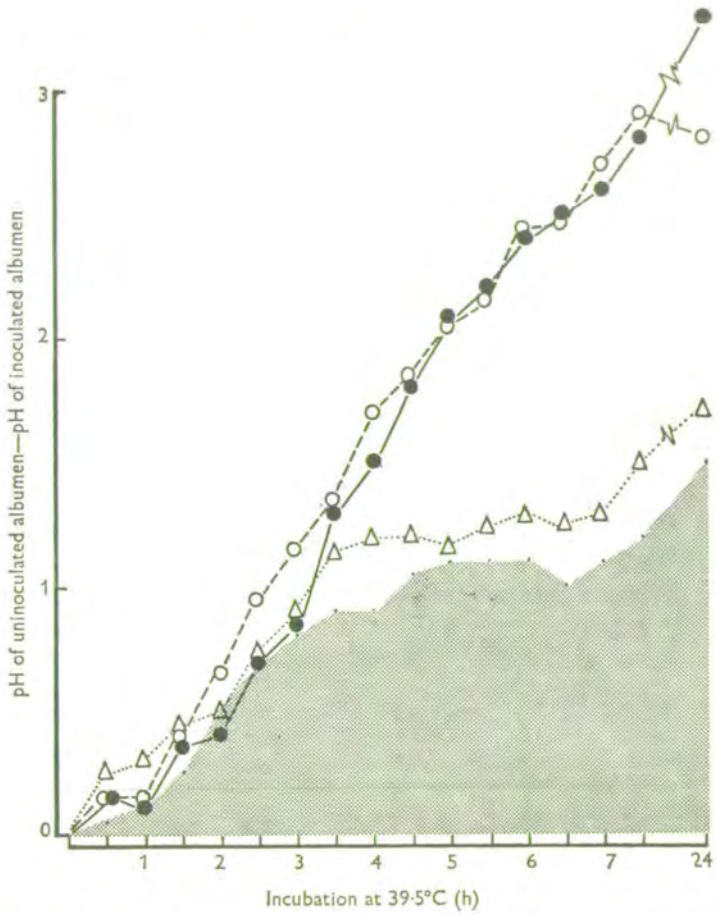

FIG. 8.—The effect of additives on the acid drift in albumen seeded with a heavy suspension of *Escherichia coli*. Beakers containing 50 ml of albumen and equivalent to 162 μg dry wt *E. coli*/ml and covered with aluminium foil were held in a water bath at 39·5 °C. Initial pH of albumen, 8·7. Shaded area, inoculated albumen alone; open triangles, inoculated albumen containing ferric chloride (8 μM/ml); open circles, inoculated albumen containing ferrous ammonium sulphate (8 μM/ml), and closed circles, inoculated albumen containing amonium chloride (16 μM/ml).

cent in egg white (Figs 6 and 7) whereas they die in fresh serum (Bullen and Rogers, 1969; Feingold, 1969; Wilkins and Lankford, 1970; Fletcher, 1971). The latter has been attributed to the action of complement (Glynn and Milne, 1967), a substance which is absent from egg white. When it is recalled that the egg white passes via the sero-amniotic connection to the amnion and eventually the gut, air sac and lungs of the embryo (Witschi, 1956), it is tempting to suggest that lysis of Gram-negative bacteria would expose the embryo to the pharmacologically active lipopolysaccharides and lipoproteins of the cell wall of Gram-negative bacteria.

In discussions of the bacteriostatic action of transferrins there is perhaps a tendency to assume implicitly that iron-deprivation will curtail the synthesis of oxygenases and haem compounds of the respiratory pathway and that this will result in the failure of an organism to grow. Studies of micro-organisms in nutrient media

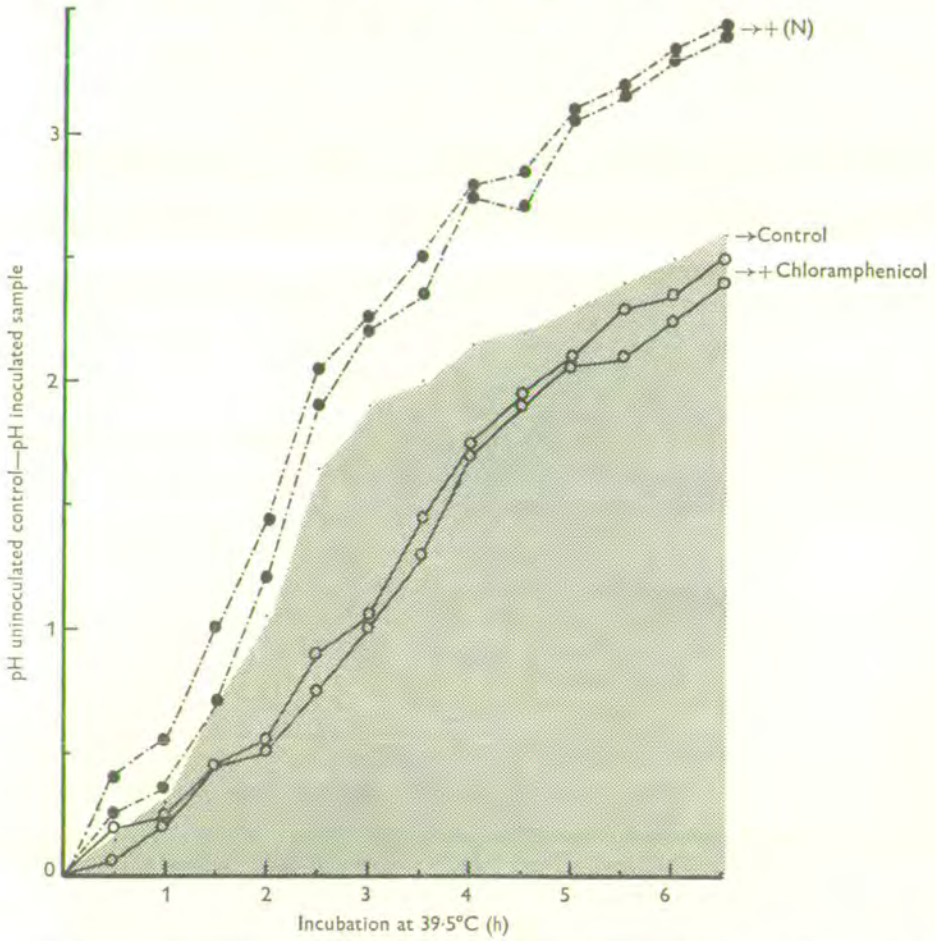

FIG. 9.—The effect of chloramphenicol on the acid drift in albumen seeded with a heavy suspension of *Escherichia coli* (equivalent to 413 μg dry wt/ml). Experimental details given in legend to Fig. 8. Initial pH of albumen, 9·05. Shaded area, inoculated albumen; closed circles, the range of readings obtained at 30 min intervals with seeded albumen containing ferrous ammonium sulphate (8 μM/ml), ferric chloride (8 μM/ml) or ferric chloride and ammonium chloride (8 μM/ml; 16 μM/ml), and open circles, the range of readings obtained with supplemented albumen containing chloramphenicol (20 mg/ml).

containing ovotransferrins (Feeney and Nagy, 1952) have shown that the unavailability of Fe^{3+} accentuates the lag phase and limits the exponential phase of growth, the extent of growth being a function of the percentage saturation of transferrins with Fe^{3+} (Theodore and Schade, 1965*a*). However, the enzymatic constitution of *St. aureus* grown in aerated medium containing glucose and unquenched transferrin (Theodore and Schade, 1965*b*) resembles that of the organism grown anaerobically in a glucose broth, the enzymes of glycolysis being abundant but those of respiration sparse (Strasters and Winkler, 1963). Such organisms increase the H-ion concentration in an aerated glucose medium containing transferrins (Theodore and Schade, 1965*b*). It is noteworthy that in the present study there was no evidence of H-ion accumulation in albumen seeded with approximately $1{\cdot}0 \times 10^5$ *E. coli* per ml and

that there was no growth in albumen held in an O_2-free atmosphere. This indicates that albumen prevents coliforms from switching from respiration to glycolysis and the evidence obtained from studies with heavy suspensions of *E. coli* in albumen suggests that the low level of non-protein-nitrogen—6·14 to 0·54 μmoles free amino acids/ml of fresh albumen (Ducay *et al.*, 1960)—is primarily responsible.

It has been suggested (Garibaldi, 1970) that organisms suspended in unsupplemented albumen would synthesise iron-transport compounds and thus ameliorate the action of transferrins. The addition of such compounds to albumen (Garibaldi, 1970) or serum (Wilkins and Lankford, 1970) does negate the action of the chelate but the results of this study as well as those of Seviour and Board (1972) indicate that they are not formed by organisms suspended in albumen.

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REFERENCES

Alderton, G., Ward, W. H. and Fevold, H. L. (1946). Identification of the bacteria-inhibiting, iron-binding protein of egg white as conalbumin. *Archs Biochem.*, **11**: 9-13.

Bullen, J. J. and Rogers, H. J. (1969). Bacterial iron metabolism and immunity to *Pasteurella septica* and *Escherichia coli*. *Nature, Lond.*, **224**: 380-382.

Ducay, E. D., Kline, L. and Mandeles, S. (1960). Free amino acid content of infertile chicken eggs. *Poult. Sci.*, **39**: 832-835.

Feeney, R. E. and Nagy, D. A. (1952). The antibacterial activity of the egg white protein conalbumin. *J. Bact.*, **64**: 629-643.

Feingold, D. S. (1969). The serum bactericidal reaction. IV. Phenotypic conversion of *Escherichia coli* from serum-resistance to serum-sensitivity by diphenylamine. *J. infect. Dis.*, **120**: 437-444.

Fletcher, J. (1971). The effect of iron and transferrin on the killing of *Escherichia coli* in fresh serum. *Immunology*, **20**: 493-500.

Garibaldi, J. A. (1960). Factors in egg white which control growth of bacteria. *Fd Res.*, **25**: 337-344.

Garibaldi, J. A. (1970). Role of microbial iron transport compounds in the bacterial spoilage of eggs. *Appl. Microbiol.*, **20**: 558-560.

Girfanova, K. N. (1949). Changes in the bactericidal properties of natural albumen and lysozyme of the chicken's egg. *Dokl. Akad. Nauk SSSR*, **68**: 1105-1108.

Glynn, A. A. and Milne, C. M. (1967). A kinetic study of the bacteriolytic and bactericidal action of human serum. *Immunology*, **12**: 639-000.

Korotkova, G. P. (1957). Antibiotic properties of chicken egg albumen. *J. gen. Chem. U.S.S.R.*, **17**: 275-285.

Miles, A. A. and Misra, S. S. (1938). The estimation of the bactericidal power of blood. *J. Hyg., Camb.*, **38**: 732-745.

Mouchan, N. A. (1962). Antimicrobial properties of albumen membranes of chickens, ducks and guinea fowl. *Vest. leningr. gos. Univ. Ser. Biol.*, **3**: 54-65.

New, D. A. T. (1956). The formation of sub-blastodermic fluids in hens' eggs. *J. Embryol. exp. Morph.*, **4**: 221-227.

Schade, A. L. (1963). Significance of serum iron for the growth, biological characteristics and metabolism of *Staphylococcus aureus*. *Biochem. Z.*, **338**: 140-148.

Schade, A. L. and Caroline, L. (1944). Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science, N.Y.*, **100**: 14-15.

SCHADE, A. L. AND CAROLINE, L. (1946). An ion binding component in human blood plasma. *Science, N.Y.*, **104**: 340-341.

SEVIOUR, E. M. AND BOARD, R. G. (1972). Bacterial growth in albumen taken from the eggs of domestic hens and waterfowls. *Br. Poult. Sci.*, **13**: 557-575.

SEVIOUR, E. M., SYKES, F. R. AND BOARD, R. G. (1972). A microbiological survey of incubated eggs of chickens and waterfowl. *Br. Poult. Sci.*, **13**: 549-556.

STRASTERS, K. C. AND WINKLER, K. C. (1963). Carbohydrate metabolism of *Staphylococcus aureus*. *J. gen. Microbiol.*, **33**: 213-231.

THEODORE, T. S. AND SCHADE, A. L. (1965*a*). Growth of *Staphylococcus aureus* in media of restricted and unrestricted inorganic iron availability. *J. gen. Microbiol.*, **39**: 75-83.

THEODORE, T. S. AND SCHADE, A. L. (1965*b*). Carbohydrate metabolism of iron-rich and iron-poor *Staphylococcus aureus*. *J. gen. Microbiol.*, **40**: 385-395.

TOKIN, B. P. (1964). Ecology of embryonic development. Immunity of embryos. *Folia biol., Praha*, **10**: 61-70.

WILKINS, T. D. AND LANKFORD, C. E. (1970). Production of *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine and its stimulation of growth in human serum. *J. infect. Dis.*, **121**: 129-132.

WITSCHI, E. (1956). *Development of Vertebrates*. London, Saunders.

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The inhibition of vegetative cell outgrowth and division from spores of *Bacillus cereus* T by hen egg albumen

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Spores of *Bacillus cereus* T germinated and formed vegetative cells in Tryptone Soya broth (TSB), pH 9·0 and 7·4 at 30°C. Spores germinated but did not form vegetative cells when suspended in hen egg white (pH 9·0) supplemented with L-alanine and inosine. Using a split image eyepiece, the volumes of germinating spores in egg white were seen to increase as a result of increases in both length and breadth. In TSB at the same pH, the major volume increase resulted from a progressive increase in cell length. Egg white supplemented with L-alanine and inosine (pH 7·6 30°C) allowed limited outgrowth to occur but the vegetative cells differed in morphology to those in TSB. $Fe(NH_4)_2(SO_4)_2.6H_2O$ overcame the inhibition of outgrowth in egg white at pH 7·8 but not in egg white at pH 9·1. Solutions containing trace elements, growth factors and casamino acids could not replace iron in this respect. Sporulation occurred in egg white only when iron was present.

Avian egg white inhibits the growth of the vegetative cells of Gram negative bacteria (Board 1964; Ayres & Taylor 1956; Seviour & Board 1972; Board & Halls 1973), fungi (Silva & Buckley 1962) and yeasts (Schade & Caroline 1944; Silva & Buckley 1962). The older literature leaves the impression that lysozyme is an important, perhaps the major, component of the egg's antimicrobial defence. This can be attributed to the work of Fleming (1922) who observed lysis of bacteria by egg white. It is notable, however, that he used organisms such as *Micrococcus lysodeikticus* which are easily lysed by this enzyme. As yet there is no evidence (Board 1969) to support the notion that lysozyme plays an important role in protecting table eggs during their storage and distribution. Of the many biologically active proteins of the egg white (Osuga & Feeney 1974) the chelator ovotransferrin is probably of much greater importance (Board & Hornsey 1979) because it deprives micro-organisms of Fe^{3+}, a property that is accentuated by the alkaline nature (pH 9–10) of avian egg whites (Sharp & Whitaker 1927). Thus, Schade & Caroline (1944) overcame the inhibition of bacterial and yeast growth by saturating ovotransferrin in egg white with Fe^{3+}.

The fate of bacterial endospores in egg white has attracted little attention. Indeed, Laschtschenko (1909), who noted the lysis of spores of *Bacillus subtilis*, appears to be the only person to study the fate of endospores in egg albumen.

This communication presents evidence that through chelation of Fe^{3+} by ovotransferrin, egg white influences the swelling of germinating spores and prevents the outgrowth of normal vegetative cells.

Materials and Methods

EGGS

Eggs of the domestic hen were stored at 4°C and used within 2 weeks of laying.

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ALBUMEN

The white of the eggs was harvested aseptically by swabbing the shell with 70% (v/v) ethanol, cracking the shell with a flamed scalpel and collecting the contents in a sterile Petri dish. The whites from several eggs were removed with sterile 10 ml wide-bore pipettes, collected in a sterile screw-capped bottle and mixed by gentle shaking. When required, the pH of the white was reduced by slowly passing a gas mixture (5% CO_2–10% O_2–85% N_2) over the surface of the egg white in a sterile Erlenmeyer flask.

ADDITIONS TO THE ALBUMEN

Germinants

To aid germination of endospores L-alanine and inosine (final concentrations 10 and 1 mmol/l, respectively) were added to egg white.

Trace element solution

This contained (per litre distilled water): NaCl 0·3 g; $(NH_4)_2SO_4$ 0·66 g; $ZnSO_4.7H_2O$ 0·11 mg; $CaSO_4.7H_2O$ 0·11 mg; $MnCl_2.4H_2O$ 0·63 mg; $MgSO_4.7H_2O$ 0·14 mg. One millilitre of this filter-sterilized (0·45 μm; Oxoid Ltd) solution was added to 25 ml of egg white.

Growth factor solution

This contained (mg/l distilled water): *p*-aminobenzoic acid 10·0; folic acid 1·0; thiamine 1·0; cyanocobalamin 1·0; nicotinic acid 1·0; pantothenic acid 1·0; riboflavin 1·0; biotin 1·0. One millilitre of this filter-sterilized solution was added to 25 ml of egg white.

Casamino acid solution

Vitamin-free casamino acids (Difco Ltd) were filter-sterilized and added to egg white to give a final concentration of 10 mg/ml.

Iron solution

A solution of filter-sterilized $Fe(NH_4)_2(SO_4)_2.6H_2O$ was added to give a final concentration of 20 μg iron/ml.

MEDIA

Tryptone Soya broth (TSB) (Oxoid Ltd) was made up at a concentration of 30 g/l in 0·2 mmol/l Tris–HCl buffer (pH 7·4 or 9·0) and sterilized at 121°C/15 min.

PRODUCTION AND CLEANING OF SPORES

Bacillus cereus T was grown at 30°C on Potato Glucose Yeast Extract agar (pH 7·2) which contained (% w/v): potato extract (Difco) 0·4; glucose 0·25; yeast extract (Difco) 0·4, in enamel trays. When sporulation was complete and the sporangia had lysed, the spores were washed off the agar with ice-cold distilled water, washed a further six times and harvested by repeated centrifugation (16 000**g**). Suspensions were cleaned of vegetative cells and debris by discarding the uppermost layers of the pellets obtained by centrifugation. The clean spore suspensions were stored at −20°C.

EXPERIMENTAL TECHNIQUE

To ensure rapid germination, spore suspensions were activated by heating at 70°C/30 min before inoculation into egg white and TSB, to give a final concentration of 10^6 spores/ml. Portions (25 ml) of TSB and egg white (supplemented with L-alanine and inosine) were incubated with gentle shaking (60 shakes/min) in Erlenmeyer flasks in a water-bath at 30°C. Immediately and at regular intervals after inoculation and mixing, a 1 ml sample was removed and mixed with 4 ml of saline–formaldehyde (1·5%–4% w/v) to arrest further spore germination and outgrowth.

MICROSCOPY

Germination

A drop of the spore–saline–formaldehyde suspension was placed on a clean microscope slide and viewed by phase-contrast microscopy. Three hundred spores, present in ten randomly selected fields of view, were scored as phase bright or phase dark and the ratio of germinated (dark) to ungerminated (bright) spores expressed as a percentage.

Length and breadth of the spores

A vernier scale image-splitting eyepiece (Vickers Instruments Ltd) was used and the lengths and breadths of 100 spores in five fields of view measured.

Outgrowth

There are five stages in the outgrowth of vegetative cells from spores of *B. cereus* T (see Fig. 3). The relative percentages of these stages in the population were determined by counting 200 of the spore/vegetative forms.

Morphology

Vegetative cells that had outgrown from spores were examined for shape, size, presence of spores, lipid granules (Burdon 1946) and Gram reaction.

STATISTICAL ANALYSIS

The mean length and breadth of the spores in egg white and TSB (both at pH 9·0 and 30°C) were calculated from 100 samples, in each case the standard deviations were determined and the lengths and breadths in the two media compared using Student's *t* test.

Results

Eighty per cent of the spores suspended in TSB (pH 9·0) and hen egg white (pH 9·0), supplemented with L-alanine and inosine, germinated within 20 min. The rate of germination was the same in both media.

SWELLING

The increases in the lengths and breadths of spores germinating in egg white (pH 9·0), supplemented with L-alanine and inosine, were greater than those in TSB at the same pH (Table 1; Figs 1a & b). With the latter, the breadth of the spores did not increase significantly after 45 min but the length increased markedly (Table 1) until cell outgrowth occurred. In egg white, however, there was an increase in both length and breadth from 60 min onwards as the germinating spore entered a second phase of swelling resulting in a 'balloon-like' form. Both vegetative cell formation in TSB and the second phase of swelling in egg white occurred at approximately the same time indicating that, in egg white, growth continued but outgrowth was inhibited. The exact cause of this swelling is unknown but apart from a small percentage (about 2%) none of these swollen forms proceeded to vegetative cell formation.

Changes in the volume of spores in TSB (pH 9·0) and egg white (pH 9·0) supplemented with L-alanine and inosine are shown in Fig. 2. The volume of the spores in the latter continued to increase for about 4 h but no free vegetative cells were seen. Vegetative cell outgrowth from the spores in TSB occurred after about 70–80 min.

Table 1. Lengths and breadths of *Bacillus cereus* T spores in Tryptone Soya broth and hen egg white (pH 9·0)

Time (min)	Tryptone Soya broth	Egg white	*t* value
0	ML† 0·6818 ± 0·1290	0·6606 ± 0·0942	−1·3223*
	MB 0·4359 ± 0·0795	0·4097 ± 0·0677	−2·5270**
10	ML 0·8069 ± 0·1010	0·8764 ± 0·1420	3·9850***
	MB 0·5688 ± 0·0683	0·6137 ± 0·0621	3·4510***
20	ML 0·8204 ± 0·1230	0·9594 ± 0·1440	7·3434***
	MB 0·6044 ± 0·0744	0·6681 ± 0·0635	6·5085***
30	ML 0·8819 ± 0·1290	0·9673 ± 0·1320	4·6240***
	MB 0·6547 ± 0·0718	0·6827 ± 0·0620	2·9610***
45	ML 0·9448 ± 0·1740	0·9898 ± 0·1360	2·0409****
	MB 0·6573 ± 0·0807	0·7023 ± 0·0623	4·4196***
60	ML 1·0927 ± 0·1950	1·1679 ± 0·1950	2·7269**
	MB 0·6797 ± 0·0619	0·7854 ± 0·0805	10·4190***

† ML = mean length; MB = mean breadth; both in μm.
* $P < 0·10$; ** $P < 0·005$; *** $P < 0·0005$; **** $P < 0·01$; $n = 100$.

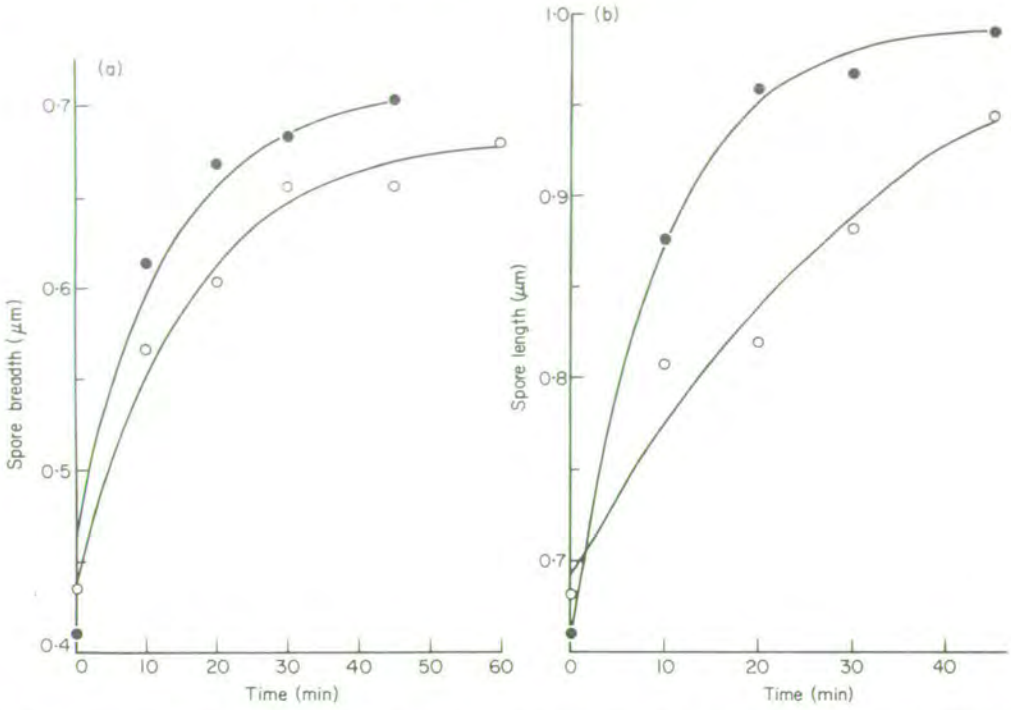

Fig. 1. Increases in (a) breadth and (b) length of spores suspended in ●, egg white; and ○, Tryptone Soya broth (both at pH 9·0). The curves were fitted from the equation: $y - B = A(1 - e^{-kt})$, where $A = y$ value as t tends to ∞ and $B = y$ at $t = 0$.

VEGETATIVE CELL OUTGROWTH

The germinated spores of *B. cereus* T in TSB passed through five stages (Fig. 3) resulting in free vegetative cells. Samples taken after 60 min in TSB (pH 7·4 and 9·0) showed a progressive shift from the fully germinated spore (stage a, see Fig. 3), to the free vegetative form (stage e).

There was very little vegetative cell formation in egg white (pH 9·0 and 7·6) supplemented with L-alanine and inosine, even after 3 h incubation (Fig. 3). The cells appeared structurally weak and they were always associated with the spore coat. If left overnight, however, some of the spores in egg white (pH 7·6) formed free vegetative cells but these differed from those in TSB in that the

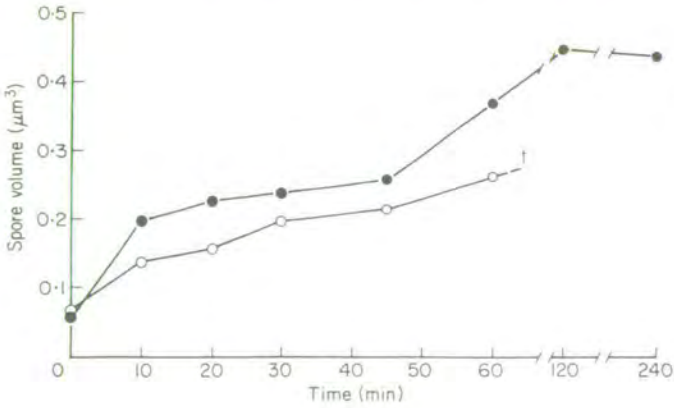

Fig. 2. Increase in volume of spores suspended in ●, egg white; and ○, Tryptone Soya broth (both at pH 9·0). The volume of the spores was calculated on the assumption that the geometry of a spore is a prolate spheroid. † Outgrowth occurred.

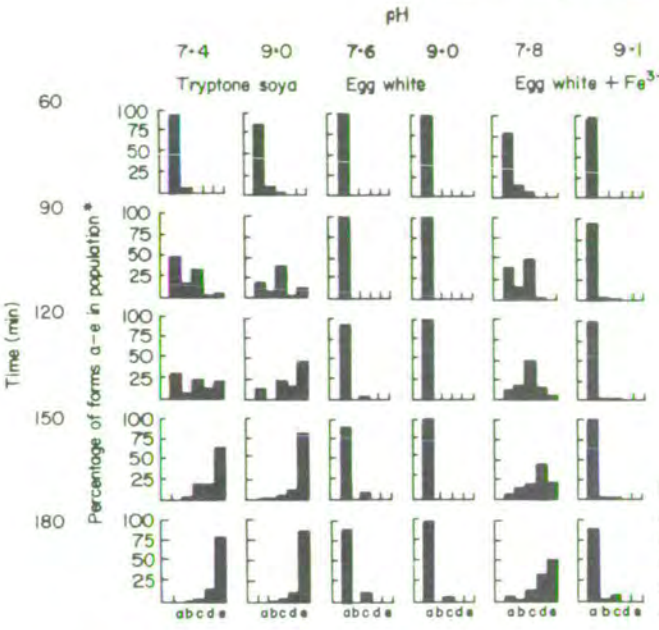

Fig. 3. Stages in the outgrowth* of *Bacillus cereus* T from spores. (a) swollen spore; (b) elongated swollen spore; (c) emerging vegetative cell; (d) emerging vegetative cells showing division; (e) free vegetative cells.

size and shape were irregular and there was no sign of spore formation unlike those from spores in TSB. The cells retained their Gram reaction and contained a small amount of lipid in granule form. Casamino acids, growth factors or trace elements added to egg white (pH 7·6) supplemented with L-alanine and inosine had no effect on the inhibition of outgrowth, however, at this pH iron removed the inhibition and allowed extensive outgrowth to take place. The cells formed in this case had a similar morphology to those that emerged from spores in a rich medium such as TSB. The cells were similar in size and shape but appeared very granular due to the presence of large amounts of lipid. They stained Gram positive and many of the cells contained spores when left overnight. Iron had no effect on the inhibition of outgrowth by egg white (pH 9·1) supplemented with L-alanine and inosine.

Discussion

In most biological fluids near pH 7 and in equilibrium with atmospheric oxygen, Fe^{2+} will be oxidized to Fe^{3+} which is readily hydrolysed to form polynuclear complexes of extremely low solubility (Spiro & Saltman 1969). Such iron is unavailable to micro-organisms unless they are able to solubilize the element from these polymers by the formation of powerful chelates, the siderophores (Snow 1970; Neilands 1972; Lankford 1973). The synthesis of these chelates is enhanced by iron-deficient conditions and in most cases they are excreted into the medium where they complex and solubilize iron present before being taken back into the cell by specific transport systems (Cox *et al.* 1970; Langman *et al.* 1972; Kadner & Bassford 1978).

Avian egg white, by virtue of its content of ovotransferrin is essentially an iron-deficient medium, a condition accentuated by its high alkalinity. In order to grow in this environment, micro-organisms would have to compete with this protein for the iron present. Garibaldi (1970; 1971; 1972) suggested that microbial iron-transport compounds play a significant role in reversing the bacteriostatic action of ovotransferrin and that the synthesis of these compounds is affected at higher growth temperatures.

Oram & Reiter (1968) showed that lactoferrin, the iron-binding protein of milk, which is similar in its action to ovotransferrin, inhibited the outgrowth from spores of *B. stearothermophilus* and *B. subtilis*. These observations confirmed the earlier work of Busta (1966) and Ashton & Busta (1967). Both sets of workers showed that iron could overcome the inhibition. The majority of their work was done using nutrient agar in Petri dishes seeded with the spores and inhibitor added to a well in the agar or in a paper disc. Thus, the conditions differed from those of spores suspended in egg white because of the differences in

the diffusion and nutritional characteristics of the two systems.

It has been shown in this study that of the three main stages in *B. cereus* T spore development—germination; swelling; outgrowth (Hitchins *et al.* 1963)—only the last two stages are affected by hen egg white. The second phase of swelling that occurs with spores suspended in egg white (pH 9·0) supplemented with L-alanine and inosine is possibly due to the synthesis of vegetative cell material; however, because outgrowth is inhibited the spore increases in volume as a consequence. It is also noteable that a marked swelling sometimes occurs when outgrowth is inhibited by other chelating agents such as ethyl picolinate (Pandey & Solanki 1980) and polyphosphates (Gould 1964).

In the same way that saturation of ovotransferrin with iron relieves the inhibition of bacterial growth in egg white (Garibaldi 1960; Board 1964), so iron relieves the inhibition of vegetative outgrowth from *B. cereus* T spores, but only when the pH of the egg white is lowered from 9·0 to 7·9. It would appear then that both the high pH of the egg white and its iron-deficient state are responsible for inhibition of outgrowth. Any vegetative cells that emerge from spores in egg white (pH 7·6) supplemented with L-alanine and inosine appear to have problems such as unequal division and growth which are overcome by the addition of iron so that although the exact mechanism of inhibition is unknown, iron does appear to be involved at some stage during outgrowth.

As growth proceeds the physical and chemical requirements become identical to those for normal vegetative growth. Just if, and how soon, the vegetative form can synthesize iron-transport compounds is unknown. There is no evidence that these compounds are present in bacterial endospores; thus, it is probably the poor capacity for obtaining iron that interferes with the further development of the swollen and newly emergent cells. It was incidentally noticed that spore formation within vegetative cells in egg white occurred only when iron was present suggesting a possible requirement for this ion for sporulation.

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References

ASHTON, D.H. & BUSTA, F.F. 1967 Milk components inhibitory to *Bacillus stearothermophilus*. *Journal of Dairy Science* **51**, 842–847.

AYRES, J.C. & TAYLOR, B. 1956 Effect of temperature on microbial proliferation in shell eggs. *Applied Microbiology* **4**, 355–359.

BOARD, R.G. 1964 The growth of Gram negative bacteria in the hen's egg. *Journal of Applied Bacteriology* **27**, 350–364.

BOARD, R.G. 1969 The microbiology of the hen's egg. *Advances in Applied Microbiology* **11**, 245–281.

BOARD, R.G. & HALLS, N.A. 1973 The effect of iron on the growth of *Escherichia coli* in albumen taken from the hen's egg. *British Poultry Science* **14**, 357–369.

BOARD, R.G. & HORNSEY, D.J. 1979 Plasma and egg white proteins. In *Chemical Zoology* Vol. 10, ed. Florkin, M. & Scheer, B.J. London: Academic Press.

BURDON, K.L. 1946 Fatty material in bacteria and fungi revealed by staining dried, fixed, slide preparations. *Journal of Bacteriology* **52**, 665.

BUSTA, F.F. 1966 Milk component(s) inhibitory to *Bacillus stearothermophilus*. *Journal of Dairy Science* **49**, 751–756.

COX, G.B., GIBSON, F., LUKE, R.K.J., NEWTON, N.A., O'BRIEN, I.G. & ROSENBERG, H. 1970 Mutations affecting iron transport in *Escherichia coli*. *Journal of Bacteriology* **104**, 219–226.

FLEMING, A. 1922 On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of the Royal Society Series B* **93**, 306–317.

GARIBALDI, J.A. 1960 Factors in egg white which control growth of bacteria. *Food Research* **25**, 337–344.

GARIBALDI, J.A. 1970 Role of microbial iron transport compounds in the bacterial spoilage of eggs. *Applied Microbiology* **20**, 558–560.

GARIBALDI, J.A. 1971 Influence of temperature on the iron metabolism of a fluorescent Pseudomonad. *Journal of Bacteriology* **105**, 1036–1038.

GARIBALDI, J.A. 1972 Influence of temperature on the biosynthesis of iron transport compounds by *Salmonella typhimurium*. *Journal of Bacteriology* **110**, 262–265.

GOULD, G.W. 1964 Effect of preservatives on the growth of bacteria from spores. In *Microbial Inhibitors in Foods* ed. Molin, N., Stockholm: Almquist & Wiksell.

HITCHINS, A.D., GOULD, G.W. & HURST, A. 1963 The swelling of bacterial spores during germination and outgrowth. *Journal of General Microbiology* **30**, 445–453.

KADNER, R.J. & BASSFORD, P. 1978 The role of the outer membrane in active transport. In *Bacterial Transport* ed. Rosen, B.P. New York: Marcel Bekker Inc.

LANGMAN, L., YOUNG, I.G., FROST, C.E., ROSENBERG, H. & GIBSON, F. 1972 Enterochelin system of iron transport in *Escherichia coli*. Mutations affecting

ferric-enterochelin esterase. *Journal of Bacteriology* **112**, 1142–1149.

LANKFORD, C.E. 1973 Bacterial assimilation of iron. *Critical Reviews in Microbiology* **2**, 273–331.

LASCHTSCHENKO, P. 1909 Uber die Keumtotende and entwicklunaphemmende Wirkung huhnere Weiss. *Zütschrift für Hygiene* **64**, 419–427.

NEILANDS, J.B. 1972 Evolution of biological iron binding centres. *Structure Bonding* **11**, 145–170.

ORAM, J.D. & REITER, B. 1968 Inhibition of bacteria by lactoferrin and other iron binding agents. *Biochimica et Biophysica Acta* **170**, 351–365.

OSUGA, D.T. & FEENEY, R.E. 1974 Avian egg whites. In *Toxic Constituents of Animal Feedstuffs* ed. Liener, I.E. New York: Academic Press Inc.

PANDEY, N.K. & SOLANKI, S. 1980 Effect of ethyl picolinate on the germination of *Bacillus cereus*: Morphological changes. *FEMS Microbiology Letters* **8**, 117–119.

SCHADE, A.L. & CAROLINE, L. 1944 Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science, New York* **100**, 14–15.

SEVIOUR, E.M. & BOARD, R.G. 1972 Bacterial growth in albumen taken from the eggs of domestic hens and waterfowl. *British Poultry Science* **13**, 557–575.

SHARP, P.F. & WHITAKER, R. 1927 The relation of the hydrogen ion concentration of egg white to its germicidal action. *Journal of Bacteriology* **14**, 17–46.

SILVA, M. & BUCKLEY, H.R. 1962 Activity of egg white against fungi pathogenic to man. In *Symposium Section of Microbiology* Vol. 77, pp. 277–291. New York Academy of Medicine.

SNOW, G.A. 1970 Mycobactins: iron-chelating growth factors from Mycobacteria. *Bacteriological Reviews* **34**, 99–125.

SPIRO, T.G. & SALTMAN, P. 1969 Polynuclear complexes of iron and their biological implications. *Structure Bonding* **6**, 116–156.

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The influence of incubation temperature and pH on the antimicrobial properties of hen egg albumen

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Gram negative bacteria, including lysozyme-resistant strains, and yeasts were killed in hen egg albumen with or without iron at 30 of 39·5°C. The albumen was more toxic at 39·5°C than at 30°C for Gram negative bacteria. With the exceptions of *Pseudomonas fluorescens*, *Acinetobacter* sp. and *Proteus vulgaris*, iron caused the growth of Gram negative bacteria or protected them from being killed in hen albumen at 39·5°C. At this temperature, however, maximal growth of and glucose utilization by *Escherichia coli* C20 only occurred in albumen supplemented with growth factors, trace metals, additional nitrogen and sufficient iron to quench ovotransferrin. The bactericidal properties of albumen could be negated by changing its pH from 9·0 or above to 7·5 or below. At 39·5°C, enterochelin allowed growth of *E. coli* in albumen at pH 7·9, but not at 9·4, whereas iron allowed growth at both pH values.

The contents of the cleiodoic egg of birds are shielded from infection by the shell and shell membranes and protected against microbial colonisation by the albumen (Board & Fuller 1974). Albumen is considered to be an unfavourable medium for microbial growth (Board & Hornsey 1978; Tranter & Board 1983) because it contains glycoproteins which have the following activities (Osuga & Feeney 1974): lysozyme causes the lysis of eubacterial cell walls (Fleming 1922); ovotransferrin chelates metal ions, especially Fe^{3+} (Tan & Woodworth 1969); avidin combines with biotin (Eakin *et al.* 1940) and ovoflavoprotein sequestors riboflavin (Rhodes *et al.* 1958, 1959). In addition, the pH of albumen (9·0–10·0) is inimical to many micro-organisms (Sharp & Whitaker 1927) and accentuates Fe^{3+}-chelation by ovotransferrin. Indeed, there is consensus of opinion that ovotransferrin and alkalinity are primarily responsible for the failure of vegetative bacterial cells to grow in albumen (Board & Halls 1973). Moreover, a recent study has shown that ovotransferrin inhibits outgrowth of cells from germinated endospores of *Bacillus cereus* T (Tranter & Board 1982).

It is well known (Lankford 1973; Neilands 1976) that when certain micro-organisms are deprived of available iron, they synthesize specific iron-chelating agents in an attempt to satisfy their requirements. Claims have been made that such compounds may be produced by bacteria suspended in albumen (Garibaldi 1970), but that their synthesis may be affected by high incubation temperatures (Garibaldi 1971, 1972). The effectiveness of enterochelin - the major iron-chelating agent of the Enterobacteriaceae - is diminished by alkaline hydrolysis (O'Brien *et al.* 1971). Although eggs intended for human consumption or used in the incubation industry will be subjected to a fairly wide temperature range (e.g. 4–37°C), there has been only one study of the influence of temperature on the antimicrobial properties of albumen (Ayres &

Taylor 1956). As this study was concerned only with changes in the population of micro-organisms in albumen, it provided no clues about the possible synthesis or effective functioning of microbial iron-chelating agents. This communication presents the results of a study of the effect of temperature and alkalinity on the response of micro-organisms to the action of enterochelin in albumen *in vitro*.

Materials and Methods

EGGS

Eggs of domestic hens were stored at 4°C and used within 2 weeks of laying.

ALBUMEN

Albumen was harvested aseptically by swabbing the shells with 70% (v/v) ethanol, cracking the shells with a flamed scapel and collecting the contents in a Petri dish. It was collected in a sterile 10 ml wide-bore pipette; that of several eggs was put into a sterile screw-capped bottle and mixed with gentle shaking. When required, the pH was reduced by slowly passing a filter-sterilised (Ferris air filters: BDH Ltd) gas mixture (5% Co_2 : 10% O_2 : 85% N_2) over the surface of the albumen in a sterile Erlenmeyer flask.

ADDITIONS TO THE ALBUMEN

Iron solutions

Solutions of Analar grade $FeSO_4.7H_2O$, $Fe(NH_4)_2(SO_4)_2.6H_2O$ or $FeCl_3.6H_2O$ (BDH Ltd) were filter-sterilized (0·45 μm; Oxoid Ltd) and added to hen albumen to give a final concentration of 20 μg iron/ml. Fresh solutions were made up immediately before use in order to minimize precipitation of ferric iron.

Iron transport compounds

Enterobactin, produced by *Escherichia coli* AN263 and prepared by the method of Young 1976), was filter-sterilized and added to egg white (final concentration, 10 mg/ml).

Casamino acid solution

Vitamin-free casamino acids (Difco) were filter-sterilized and added to egg albumen to give a final concentration of 10 mg/ml.

Growth factor solution

This contained (mg/l distilled water): *p*-aminobenzoic acid, 10·0; folic acid, 1·0; thiamine, 1·0; cyanocobalamin, 1·0; nicotinic acid, 1·0; pantothenic acid, 1·0; riboflavin, 1·0; biotin, 1·0. One millilitre of this filter-sterilized solution was added to 25 ml of albumen.

Trace element solution

This contained (per litre distilled water); NaCl, 0·3 g; $(NH_4)_2SO_4$, 0·66 g; $ZnSO_4.7H_2O$, 0·11 mg; $CaSO_4.7H_2O$, 0·11 mg; $MnCl_2.4H_2O$, 0·63 mg; $MgSO_4.7H_2O$, 0·14 mg. One millilitre of the filter-sterilized solution was added to 25 ml albumen.

Nitrogen sources

NH_4Cl, $(NH_4)_2SO_4$, KNO_3 (BDH Ltd) were filter-sterilized and added to albumen (final concentration, 1 mg/ml).

MICRO-ORGANISMS

Bacteria

Escherichia coli strains, K4208, C3650, C20; *Salmonella* serotypes, *waycross*, *brandenburg*, *dublin*; *Enterobactor aerogenes*, *Proteus vulgaris*, *Alcaligenes faecalis*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Brochothrix thermosphacta* and *Kurthia zopfii* were obtained from the stock culture collection of the Microbiology Department, Bath University.

Pseudomonas aeruginosa, *Ps. fluorescens*, *Acinetobacter* sp., *Bacillus cereus* T, *B. megaterium*, *Streptococcus faecalis* and *Staphylococcus aureus* were donated by Professor G.W. Gould from the stock culture collection at Unilever Limited, Colworth House, Sharnbrook, Bedfordshire.

Escherichia coli 0111 and *E. coli* 0141 were the kind gift of Professor H.J. Rogers, National Institute for Medical Research, Mill Hill, London.

All cultures were stored on Nutrient Agar (Oxoid) slopes at 4°C; they were subcultured and their purity checked every four weeks.

Yeasts

Bretanomyces anomalus, *Candida valida*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* were the kind gift of Dr R.R. Davenport, Long Ashton Research Centre, Long Ashton, Bristol. They were maintained at 4°C on Yeast Extract Peptone Glucose Agar (YEPG) slopes containing (g/l): yeast extract (Difco), 10·0; peptone (Difco), 20·0; D-glucose, 20·0; agar, 20·0. The cultures were subcultured and their purity checked every four weeks.

EXPERIMENTAL TECHNIQUE

Inocula were prepared by centrifuging (1000 g) a culture in Tryptone Soya Broth (Oxoid) which had been incubated at 39·5°C in a shaking water bath for 18 h and washing the pellet of cells with two changes of Ringer solution. The final suspension of cells was diluted to an appropriate level of *ca* 10^4–10^5 cells/ml with Ringer's solution and 1 ml added to 25 ml of albumen in 250 ml Erlenmeyer flasks which had been incubated in a shaking (60 cycles/min) water bath overnight at the temperatures stated in the text. Samples of albumen (1 ml) were serially diluted in 9 ml amounts of Ringer solution. Sixteen 0·02 ml amounts of each solution were placed individually on the dried surface of Nutrient Agar (Oxoid) and the colonies counted after 24 and 48 h at 37°C.

The counting of viable yeast cells was based on the method of Pringle & Mor (1975). The serial dilutions were sonicated for 1 min at an amplitude of 6·0 μm peak-to-peak on a sonicator (MSE Instruments Ltd) in order to break up clumps of cells. One millilitre of each dilution was used to prepare pour plates using YEPG agar. The colonies were counted after 24 and 48 h at 30°C. The plate-dilution frequency technique (Harris & Sommers 1968) was used for bacteria, *Pr. vulgaris*, *B. cereus* T, that did not form small discrete colonies on solid media.

Glucose determination

Glucose concentrations were determined using the Boehringer blood sugar kit (Boehringer-Mannheim). A sample (0·1 ml) of albumen was precipitated in 1·0 ml 0·16% (w/v) uranyl acetate solution. The protein was pelleted by centrifuging (5000 g) and 0·2 ml of the supernatant used for the assay.

Results

TEMPERATURE

Gram positive bacteria

Gram positive bacteria in hen egg albumen (Fig. 1) were killed at 30°C or 39·5°C irrespective of whether or not iron was present at a concentration sufficient to saturate the ovotransferrin. As we found two of the organisms, *B. cereus* T and *M. luteus*, were resistant to the lytic action of purified hen egg white lysozyme, the death of Gram positive bacteria in albumen could not be attributed solely to this enzyme.

Gram negative bacteria

Of the Gram negative bacteria inoculated into unsupplemented hen egg white at 30°C (Fig, 2), *Acinetobacter* sp. was the only organism which failed to give a viable count greater than 10^2 cells/ml; it was also the only one that did not multiply in albumen in which ovotransferrin had been saturated with iron. The populations of the majority of Gram negative bacteia remained static or declined slightly in size in unsupplemented albumen at 30°C, but those of *Salm. brandenburg* and *Salm. dublin* increased slightly.

The populations of all of the Gram negative bacteria decreased in size in unsupplemented albumen at 39·5°C, very few cells being present in albumen incubated for 24 h. The addition of sufficient iron to quench the chelating potential of ovotransferrin either led to the growth of the bacteria or, with the exception of *Pr. vulgaris*, *Ps. fluorescens and Acinetobacter* sp., protected them from death, The death of the last two mentioned organisms was probably due to temperature alone. As *Pr. vulgaris* requires nicotinic acid for growth, the absence of this vitamin in egg white probably contributed to its death. It was noteworthy that with *E. coli* C20, an organism that has no requirement for vitamins and organic sources of combined nitrogen, maximal growth and glucose utilization were obtained only in albumen supplemented with trace metals, additional combined nitrogen and

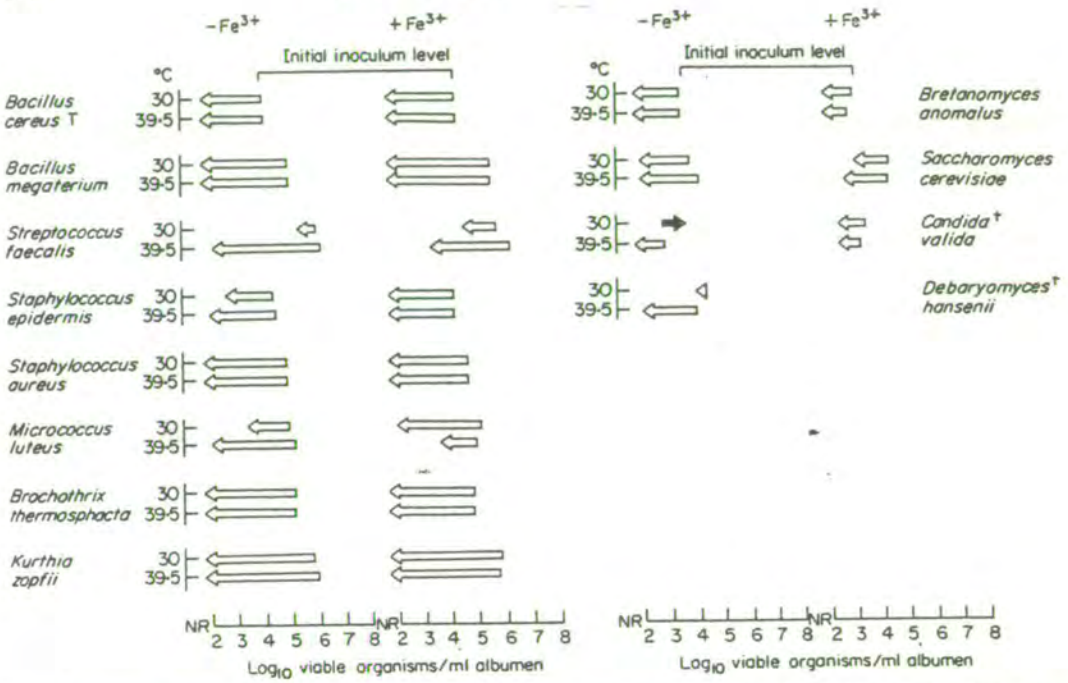

Fig. 1. The effect of iron on the survival of Gram positive bacteria and yeast vegative cells in hen egg albumen held at 30°C or 39·5°C for 24 h. Open arrows show death of the organism; closed arrows show growth of the organism; ND, viable organisms were not detected in the albumen. †Incubated for 10 h only.

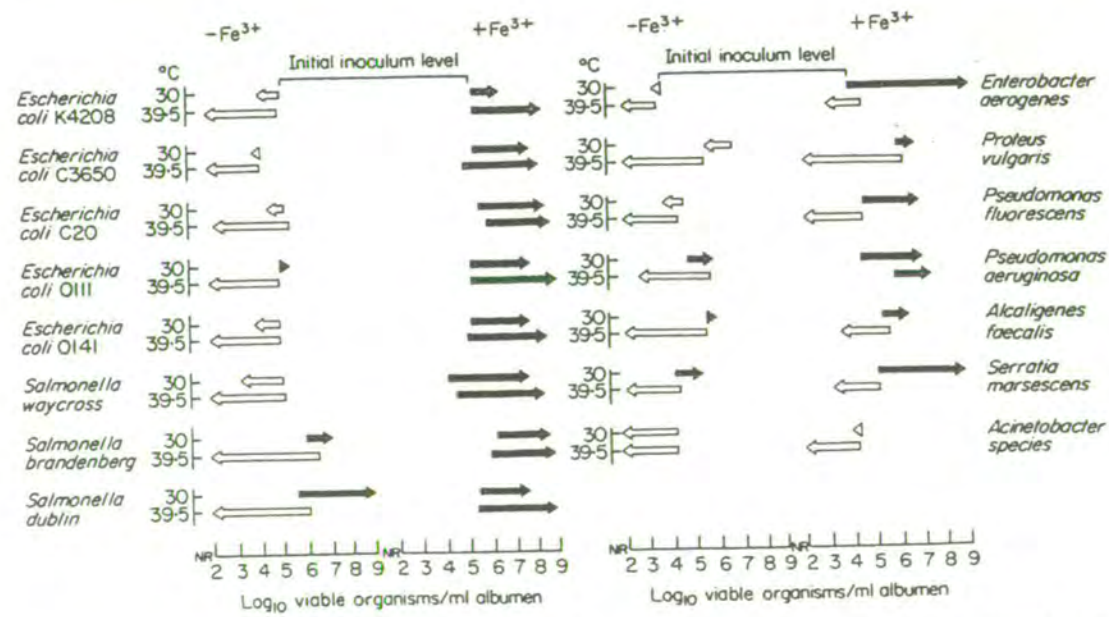

Fig. 2. The effect of iron on the survival of Gram negative bacteria in hen egg albumen held at 30°C or 39·5°C for 24 h. Open arrows show death of the organism, closed arrows show growth of the organism; ND, viable organisms were not detected in the albumen.

vitamins as well as iron sufficient to saturate ovotransferrin (Fig. 3).

Additional evidence that incubation temperature influences the response of Gram negative bacteria in egg white was obtained when *E. coli* C3650 was incubated in albumen over a range of temperatures (Fig. 4); the number of viable cells declined rapidly with incubation at 37, 39·5 or 44°C, but only slowly if at all, at 25 or 30°C. Indeed, in some cases the populations increased in size after 2–3 d incubation at 25 and 30°C. It is evident from Fig. 5 that the death of *E. coli* in albumen was not caused by transfer of organisms from a nutritionally complete (tryptone soya broth) to a nutritionally inadequate medium (egg white). Death rates similar to those in Fig 4 occurred in inoculated albumen that was incubated for 12 h at 25°C. The response of coliforms to incubation in albumen at 39·5°C was not influenced by the medium (complex *vs* simple) used to grow viable cells or inoculum size. Thus, it was concluded that the organisms were killed by the albumen and not by the recovery medium as a result of physiological damage suffered during incubation in the albumen.

Yeasts

With the exception of *Candida valida*, the response of the yeasts to albumen was similar to that of Gram positive bacteria. Iron-saturation of ovotransferrin did not protect these organisms from the inimical factors. In contrast to the Gram negative bacteria, however, incubation temperatures had little effect on the response of yeasts to unsupplemented albumen.

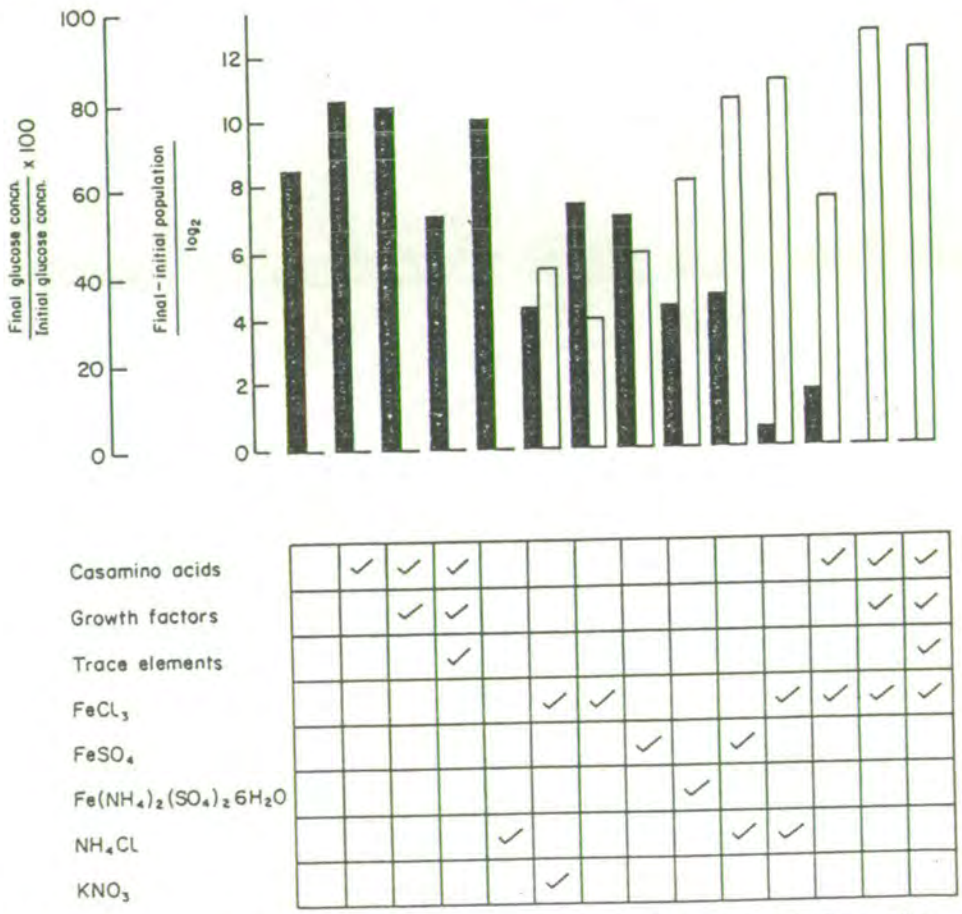

Fig. 3. The effect of various supplements added to hen egg albumen at 39·5°C on the growth and glucose utilization by *Escherichia coli* C20 over 24 h.

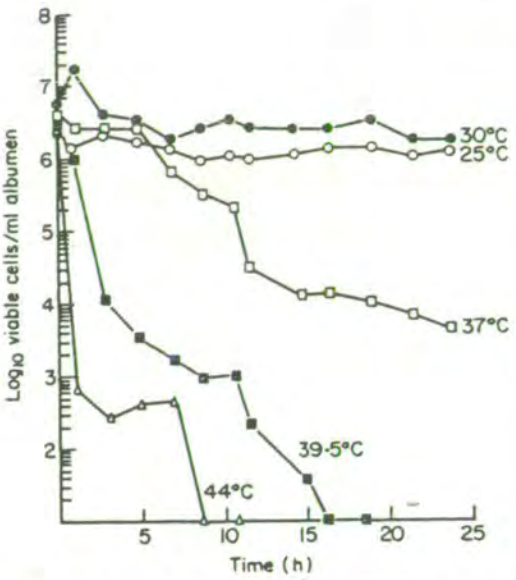

Fig. 4. The effect of incubation temperature on the fate of *Escherichia coli* C3650 in hen egg white, pH 9·2. ND, viable organisms were not detected in the albumen. ○, 25°C; ●, 30°C; □, 37°C; ■, 39·5°C; △, 44°C.

HYDROGEN ION CONCENTRATION

At 39°C unsupplemented albumen was bactericidal to strains of *E. coli* at pH values greater than 8·8, but not at values of 7·5 or less (Fig. 6). The organisms grew at both pH ranges in albumen supplemented with iron, there being no perceptible lag phase in supplemented albumen poised at pH 7·5 or less. It was notable, however, that the eventual size of the populations at the latter values were larger than those in supplemented albumen at or above pH 8·8. Analogous results were obtained when enterochelin was used to supplement albumen incubated at 39·5°C (Fig. 7), growth occurring in supplemented albumen poised at pH 7·9 but not at pH 9·4.

Discussion

Avian albumen, in common with other biological fluids such as mammalian serum and milk, contains an iron-binding protein belonging to the class known as the transferrins (Feeney & Komatsu 1966). Many investigators (Schade 1963; Rogers 1967; Bullen *et al.* 1972) have shown that inhibition of microbial growth by these proteins is due to iron deprivation. It is overcome simply by adding sufficient of this element to saturate the protein. With the exceptions of *Acinetobacter* sp. and *Ps. fluorescens*, iron saturation of ovotransferrin caused the growth of, or protected, the Gram negative bacteria used in this study from death in albumen at 30 39·5°C (Fig. 2). In the case of Gram positive bacteria, including lysozyme resistant strains and yeasts, there did not appear to be any response to the saturation of ovotransferrin with iron. This observation suggests that factors other than lysozyme and ovotransferrin are involved in the death of these organisms in albumen. Thus, for example, the inhibition of the yeasts may have been due to avidin, another chelating protein of egg white (Osuga & Feeney 1974), which makes biotin unavailable. Although this protein is present in hen albumen at much lower concentration (0·1% of the total protein) compared with ovotransferrin (13·7%), its strong affinity for biotin has provided the basis of an assay with biotin-requiring yeasts (Hertz 1943). In addition, studies of the antimicrobial properties of polymorphonuclear lymphocytes of rabbits have revealed (Gladstone & Walton 1971; Gladstone *et al.* 1974) the bactericidal nature of cationic proteins. Such proteins, which may be precipitated by the addition of iron, are also found in albumen, thus it could be that the death of micro-organisms in egg white

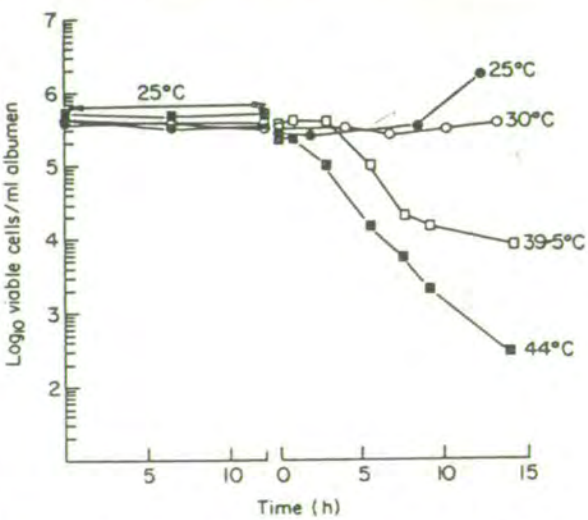

Fig. 5. The effect of incubation temperature on the fate of *Escherichia coli* C3650 in hen egg white after holding the inoculated white at 25°C for 12 h before changing the temperature. ●, 25°C; ○, 30°C; □, 39·5°C; ■, 44°C.

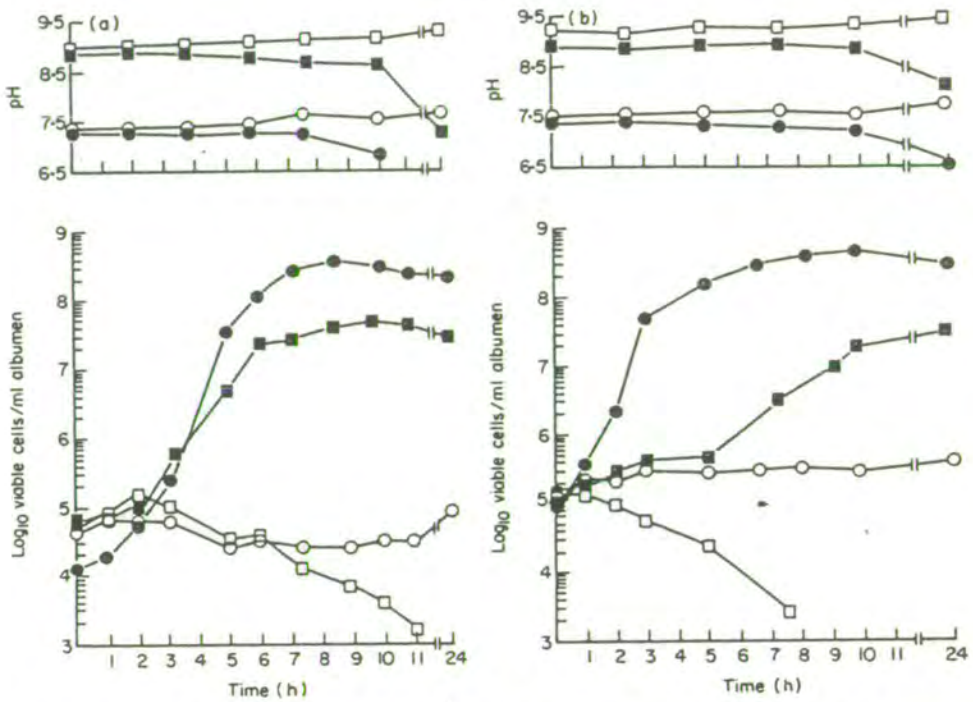

Fig. 6. The effect of egg white pH on the fate of (a) *Escherichia coli* 0141 and (b) *Escherichia coli* 0111 in hen egg white (open symbols) and iron saturated egg white (closed symbols) at 39·5°C.

may be due to an as yet uncharacterized protein.

The influence of temperature on the antimicrobial mechanisms of albumen has been neglected. Indeed, Ayres & Taylor (1956) appear to be the only workers to demonstrate that albumen is more toxic to bacteria at high rather than low temperatures. It is notable that previous workers have reported growth of bacteria in hen egg albumen *in vitro* (Garibaldi 1960; Seviour & Board 1972) and spoilage of eggs (Elliott 1954; Brown *et al.* 1966) at incubation temperatures below 30°C, while others have demonstrated death of micro-organisms or their failure to grow in albumen *in vitro* incubated at or above 37°C (Schade & Caroline 1944; Board & Halls 1973). The results in Figs. 4 and 5 suggest that differences in incubation temperatures may account for the differing results of these workers.

Bacteria that grew in unsupplemented egg white at 30°C presumably possessed a specific mechanism e.g. enterochelen production, for acquiring iron from partly saturated ovotransferrin. We failed to demonstrate the occurrence of such compounds in egg white due to the insensitivity of the assay methods. The postulated mechanism was not present, or was unable to function efficiently, at 39·5°C. It is noteworthy that Garibaldi (1971) suggested that microbial siderophores—low molecular weight iron-chelating molecules produced by micro-organisms in response to iron deprivation (Lankford 1973; Neilands 1981)—play a significant role in negating the bacteristatic action of ovotransferrin, but Garibaldi 1972) observed that the synthesis of these compounds is diminished at temperatures approaching the upper limit for the organism which he studied. His observations may account for the growth occurring in albumen incubated at 25–30°C and death at 37–44°C (Fig. 4). Thus in this respect, the antimicrobial action of hen egg albumen may be akin to mammalian serum in that a reduction in iron content coupled with an elevation of body temperature (fever) is a co-ordinated host-defence mechanism that prevents production of siderophores by invading pathogenic bacteria (Weinberg 1974; Kluger & Rothenburg 1979).

The results in this study (Figs. 6 and 7) also confirm the observation of Sharp & Whitaker

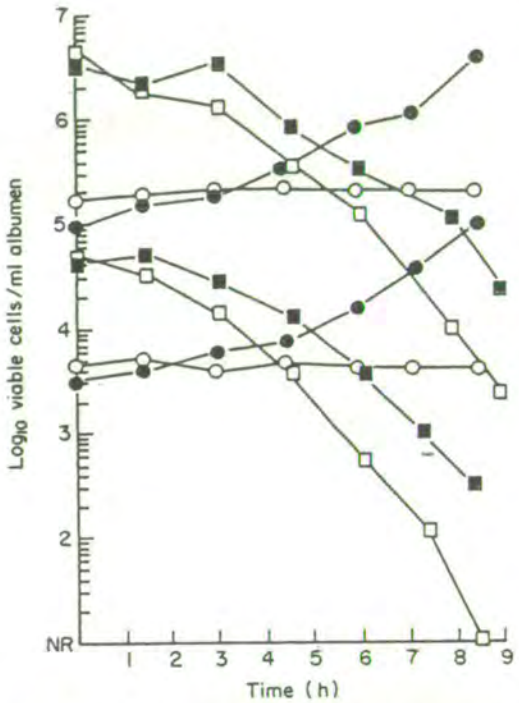

Fig. 7. The effect of adding purified enterochelin (10 mg/ml) on the fate of *Escherichia coli* 0141 in hen egg white at pH 7·9 (circles) and pH 9·4 (squares) both at 39·5°C. Open symbols, albumen alone; closed symbols, albumen plus enterochelin and 2 μg/ml iron added as $Fe(NH_4)_2(SO_4)_2.6H_2O$.

(1927) that egg albumen was bactericidal at pH 9–10 but bacteristatic at pH 6–8. In the light of the present discussion, together with the observation that iron saturation of ovotransferrin overcame the inhibition at both pH 7·2 and 9·0 (Fig. 6), it is probable that the high alkalinity of egg white interferes with bacterial iron metabolism such that bacteria are prevented from obtaining sufficient iron for growth.

The major iron-chelating siderophore of *E. coli*, enterochelin, is a cyclic triester of 2, 3-dihydroxybenzoylserine. As enterochelin is inherently unstable—it undergoes oxidation of the ring hydroxy groups and hydrolysis of the lactone at high alkaline pH (O'Brien *et al.* 1971)—its synthesis in albumen may not benefit the bacterial cell because the hydrolytic products which include 2,3-dihydroxybenzoylserine and 2,3-dihydroxybenzoic acid are only weak chelating agents (O'Brien *et al.* 1971; Hancock *et al.* 1977), the stability of the complexes formed with iron decreases with decreasing complexity of the ligand. Thus, at 39·5°C and pH 7·9, enterochelin added to albumen (Fig. 7) would be the major iron-chelating species which scavenges sufficient iron for growth suggesting that although *E. coli* 0141 was unable to synthesize or secrete its own siderophore, it was able to take up the molecules from the medium. At pH 9·4, however, the hydrolytic products of enterochelin were unable to supply sufficient iron for growth of the organism at 39·5°C and death resulted (Fig. 7). These hydrolytic products may be able to provide sufficient iron for bacteria growing at lower temperatures with a lower metabolism and reduced iron requirements (Perry & Weinberg 1973), a view which may explain the results obtained with bacteria at 30°C in egg albumen.

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References

AYRES, J.C. & TAYLOR, B. 1956 Effect of temperature on microbial proliferation in shell eggs. *Applied Microbiology* **4**, 355–359.

BOARD, R.G. & HALLS, N.A. 1973 The effect of iron on the growth of *Escherichia coli* in albumen taken from the hen's egg. *British Poultry Science* **14**, 359–371.

BOARD, R.G. & FULLER, R. 1974 Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biological Reviews* **49**, 15–49.

BOARD, R.G. & HORNSEY, D.J. 1978 Plasma and egg white proteins. In *Chemical Zoology* Vol. 10, ed. Florkin, M. & Scheer, B.J., pp. 37–74. London: Academic Press.

BROWN, W.E., BAKER, R.C. & NAYLOR, H.B. 1966 Egg spoilage as effected by route of exposure. *Poultry Science* **45**, 279–283.

BULLEN, J.J., ROGERS, H.J. & LEIGH, L. 1972 Iron binding proteins in milk and resistance to *Escherichia coli* infection in infants. *British Medical Journal* **i**, 69–75.

EAKIN, R.E., SNELL, E.E. & WILLIAMS, R.J. 1940 A constituent of raw egg white capable of inactivating biotin *in vitro*. *Journal of Biological Chemistry* **136**, 801–802.

ELLIOTT, R.P. 1954 Spoilage of shell eggs by pseudomonads. *Applied Microbiology* **2**, 158–164.

FEENEY, R.E. & KOMATSU, S.K. 1966 The Transferrins In *Structure and Bonding* Vol. 1, ed. Jorgenden, C.K., Neilands, J.B., Nyholm, R.S., Reinen, D. & Williams, R.J.P. pp. 149–206. Berlin: Springer-Verlag.

FLEMING, A. 1922 On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of the Royal Society London Series B* **93**, 306–317.

GARIBALDI, J.A. 1960 Factors in egg white which control growth of bacteria. *Food Research* **25**, 337–344.

GARIBALDI, J.A. 1970 Role of microbial iron transport compounds in the bacterial spoilage of eggs. *Applied Microbiology* **20**, 558–560.

GARIBALDI, J.A. 1971 Influence of temperature on the iron metabolism of a fluorescent pseudomonad. *Journal of Bacteriology* **105**, 1036–1038.

GARIBALDI, J.A. 1972 Influence of temperature on the biosynthesis of iron transport compound by *Salmonella typhimurium*. *Journal of Bacteriology* **110**, 262–265.

GLADSTONE, G.P. & WALTON, E. 1971 The effect of iron and haematin on the killing of staphylococci by rabbit polymorphonuclear leucocytes. *British Journal of Experimental Pathology* **52**, 452–464.

GLADSTONE, G.P., WALTON, E. & KAY, U. 1974 The effect of culture conditions from rabbit polymorphonuclear leucocytes. *British Journal of Experimental Pathology* **55**, 427–447.

HANCOCK, R.E.W., HANTKE, K. & BRAUN, V. 1977 Iron transport in *Escherichia coli* K-12:2,3-dihydroxybenzoate-promoted iron uptake. *Archives of Microbiology* **114**, 231–239.

HARRIS, R.F. & SOMMERS, L.E. 1968 Plate-dilution frequency technique for assay of microbial ecology. *Applied Microbiology* **16**, 330–334.

HERTZ, R. 1943 Modification of the yeast-growth assay method for biotin. *Proceedings of the Society for Experimental Biology and Medicine* **52**, 15–17.

KLUGER, M.J. & ROTHENBERG, B.A. 1979 Fever and reduced iron: Their interaction as a host defence response to bacterial infection. *Science* **203**, 374–376.

LANKFORD, C.E. 1973 Bacterial assimilation of iron. *Critical Reviews in Microbiology* **2**, 273–331.

NEILANDS, J.B. 1976 Microbial iron transport compounds (sideropores). In Symposium on the *Development of Iron Chelators for Clinical Use* ed. Anderson, W.T. & Hiller, M.C., pp. 5–44. Bethseda, Maryland: Department of Health, Education and Welfare Publication (NIH) 76-994.

NEILANDS, J.B. 1981 Microbial iron compounds. *Annual Review of Biochemistry* **50**, 715–731.

O'BRIEN, I.G., COX, G.B. & GIBSON, F. 1971 Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. *Biochimica et Biophysica Acta* **237**, 537–549.

OSUGA, D.T. & FEENEY, R.E. 1974 Avian egg whites. In *Toxic Constituents of Animal Foodstuffs* ed. Liener, I.E., pp. 39–71. New York: Academic Press.

PERRY, R.D. & WEINBERG, E.D. 1973 Effect of iron deprivation and temperature upon bacterial survival. *Microbios* **8**, 129–135.

PRINGLE, J.R. & MOR, J-R. 1975 Methods for monitoring the growth of yeast cultures and for dealing with the clumping problem. *Methods in Cell Biology* **11**, 131–168.

RHODES, M.B., AZARI, P.R. & FEENEY, R.E. 1958 Analysis, fractionation and purification of egg white proteins with cellulose cation exchanger. *Journal of Biological Chemistry* **230**, 399–409.

RHODES, M.B., BENNETT, N. & FEENEY, R.E. 1959 The flavoprotein-aproprotein system of egg white. *Journal of Biological Chemistry* **234**, 2054–2060.

ROGERS, H.J. 1967 Bacteriostatic effect of horse sera and serum fractions on *Clostridium welchii* type A, and the abolition of bacteriostasis by iron salts. *Immunology* **7**, 445–456.

SCHADE, A.L. 1963 Significance of serum iron for the growth, biological characteristics and metabolism of *Staphylococcus aureus*. *Biochemische Zeitschrift* **338**, 140–148.

SCHADE, A.L. & CAROLINE, L. 1944 Raw egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli* and *Saccharomyces cerevisiae*. *Science* **100**, 14–15.

SEVIOUR, E.M. & BOARD, R.G. 1972 Bacterial growth in albumen taken from the eggs of domestic hens and waterfoul. *British Poultry Science* **13**, 557–575.

SHARP, P.F. & WHITAKER, R. 1927 The relation of the hydrogen ion concentration of egg white to its germicidal action. *Journal of Bacteriology* **14**, 17–46.

TAN, A.T. & WOODWORTH, R.C. 1969 Ultraviolet difference spectral studies of conalbumin complexes with transition metal ions. *Biochemistry* **6**, 3711–3716.

TRANTER, H.S. & BOARD, R.G. 1982 The inhibition of vegetative cell outgrowth and division from spores of *Bacillus cereus* T by hen egg albumen. *Journal of Applied Bacteriology* **52**, 67–73.

TRANTER, H.S. & BOARD, R.G. 1983 The antimicrobial defence of avian eggs: Biological perspective and chemical basis. *Journal of Applied Biochemistry* (In Press)

WEINBERG, E.D. 1974 Iron and susceptibility to infectious disease. *Science* **184**, 952–956.

YOUNG, I.G. 1976 Preparation of enterochelin from *Escherichia coli*. *Preparative Biochemistry* **6**, 123–131.

The Microbiological Contamination of Egg Shells and Egg Packing Materials[1,2]

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THE microbiology of egg shells and egg packing materials has received inadequate attention. In England, Haines (1938) found levels of bacterial contamination ranging from $1.30\times10^4-8.0\times10^6$ (incubation, 20°C.) and $3.5\times10^4-1.6\times10^6$ (incubation, 37°C.) on the shells of 130 eggs, the majority of which were procured from a farm. Rosser (1942) reported average counts of 7.0×10^4 organisms per shell of Canadian market eggs and 1.03×10^5 per shell of eggs obtained from a farm. Forsythe *et al.* (1953) found an average of 6.3×10^4 (range, $1.0\times10^4-1.0\times10^6$) microorganisms on the shells of eggs produced on an experimental poultry farm. Haines (1938) examined 100 isolates from shells and found that gram-negative bacteria (38%), bacilli (30%), and gram-positive cocci (25%) were the most prevalent contaminants. He concluded that eggs were exposed to contamination from a wide variety of sources, the chief ones being feces, manure, and soil.

The present study was undertaken with the object of ascertaining the range of microbial contamination on egg shells and egg packing materials received at an egg grading station. Attention was given also to the possibility of contamination of the shells with organisms present on fillers and flats. In addition, a large number of isolates were characterized. It was anticipated that this information would indicate the major source of contamination to which eggs are exposed.

METHODS

An egg collecting and grading plant in Central Iowa was visited at two week intervals August, 1962–January, 1963 inclusive. Used fillers and flats were taken from the unloading benches after having been passed as suitable for reissue to the egg producer. Some of these materials were soiled with dried albumen or yolk material and others with dust. New fillers and flats were taken from a stock maintained in the unloading area. Each of the samples was placed in a sterile envelope for transit to the laboratory. Cotton swabs moistened with 0.1% peptone water were rubbed over an area of 10 cm.^{2} on the interior surface of the bottom of egg cases that were awaiting delivery to the farm. The swabs were submerged in 9 ml. of peptone water during transit to the laboratory. Grade A (clean), B (lightly soiled), and C (heavily soiled, cracks or checks) eggs were collected from each of the six candling stations and were placed in new 1-dozen egg cartons by the candlers. Some eggs were examined immediately on return to the laboratory and others after storage for 1 week in a refrigerated (10°C.) display cabinet. In

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most prevalent contaminants. Coliform organisms were recovered from 4 of the 33 cases and enterococci from 21 cases.

In commercial practice, the fillers and flats protect eggs from contamination with the organisms present in the case. There is a possibility, however, that these organisms may be introduced to the atmosphere when eggs are being unpacked. This possibility was investigated, and the results are shown in Figure 1. The petri dish on the left side was exposed in a small room, and the one on the right was exposed in the same room after 4 egg cases had been unpacked. It would appear that the creation of an aerosol during the unpacking of eggs may be an important source of contamination in egg-breaking plants, particularly as these commonly receive grade C eggs in containers considered too old or dirty for re-issue to the egg producer.

Figure 2 contains the results obtained from a survey of flats. In general, the lowest level of contamination was found on new flats and the highest on dirty flats. A similar trend (Table 2) was also evident in the isolation of coliforms and enterococci. Aerobic spore-forming bacilli were the dominant organisms on new flats, whereas a heterogeneous microflora was isolated from the used and dirty flats. A survey of fillers did not produce similar trends. It will be seen in Figure 3 that the majority of fillers harbored about a million microorganisms regardless of whether the fillers were new or used. The highest counts were given by the dirty fillers. The high incidence of contamination of new fillers with enterococci (Table 2) was a notable feature of this survey. Many colonies developed on plates of Barnes' (1956) medium that had been exposed to the atmosphere in the unloading area of the grading station. For this reason, it was concluded that new fillers were contaminated during storage in this area.

The flats have a mat-finish, and considerable areas of their irregular surfaces are always exposed, thus predisposing

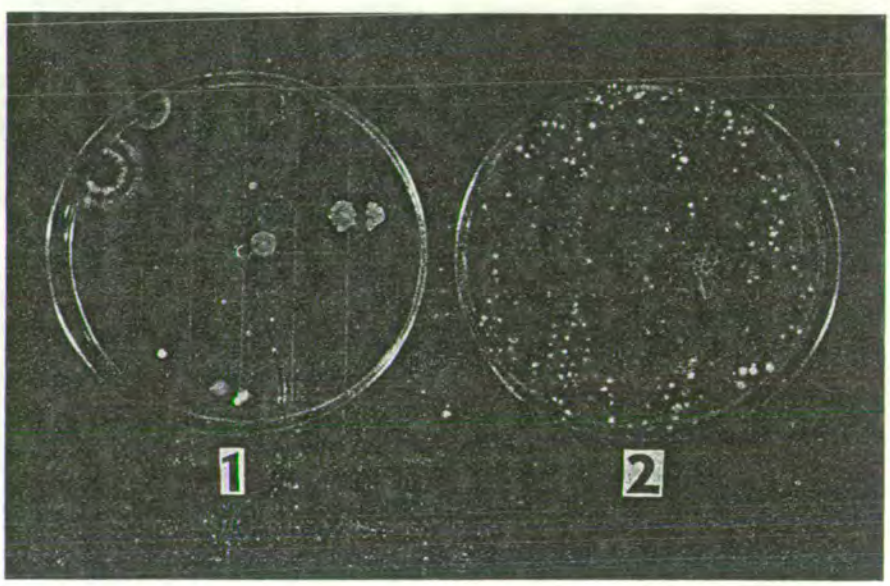

FIG. 1. Effect of unpacking on contamination of room air in egg plant. Dish No. 1, before unpacking; Dish No. 2, after 4 cases were unpacked.

TABLE 2.—*Recovery of coliforms and enterococci from fillers and flats*

		Number of samples	Percent contaminated with:	
			Coliforms	Entero-cocci
Flats	New	18	0	17
	Used	35	32	83
	Dirty	33	55	88
Fillers	New	14	0	79
	Used	34	18	82
	Dirty	29	19	100

these structures to heavy contamination. This would appear to be the reason for the trends shown in Figure 2. The fillers on the other hand, have a hard, smooth surface and, when not in use, ony a small area is exposed. This may account for the greater uniformity in the levels of contamination (Figure 3) found with new, used, and dirty fillers.

An attempt to demonstrate the transfer of organisms from dirty fillers and flats to the shell of eggs yielded the results presented in Figure 4. Neither the conditions of storage nor the state of the fillers and flats had any detectable effect on the level of contamination on the shells of naturally clean eggs.

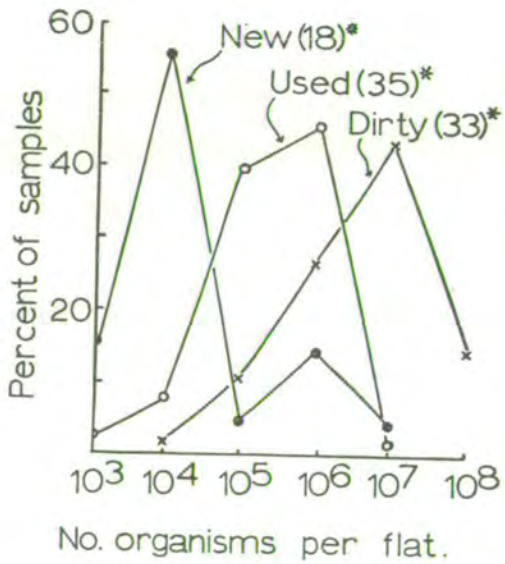

FIG. 2. Frequency distribution of contamination on flats.
* Number of samples examined.

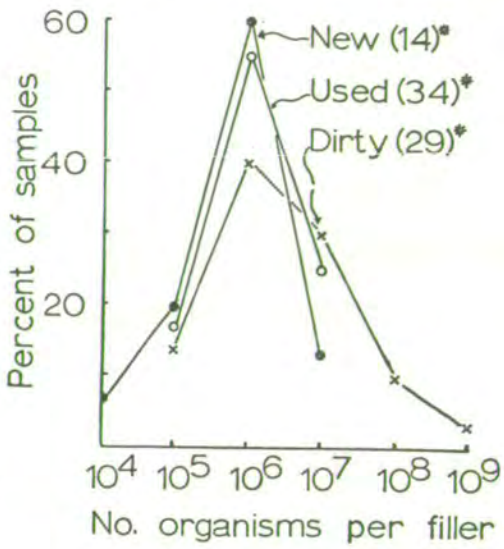

FIG. 3. Frequency distribution of contamination on fillers.
* Number of samples examined.

The level of contamination on the shells of eggs entering the grading station ranged from 10^2–10^8 microorganisms per shell. The results obtained from a survey of large clean (grade A) and lightly soiled eggs (grade B) are presented in Figure 5. The range of counts shown by these eggs was larger than those reported by Haines (1938), Rosser (1942) and Forsythe *et al.* (1953). In general, the clean eggs harbored fewer organisms per shell than did the lightly soiled eggs. Further evidence of a general relationship between the level of contamination and the cleanliness of the shell was provided by grade C eggs. In this instance (Figure 6), stained and soiled eggs had counts ranging from 10^3–10^6 organisms per shell and badly-soiled eggs harbored from 10^4–10^7 organisms per shell. Coliform organisms

TABLE 3.—*Recovery of coliforms and enterococci from the shells of eggs*

Grade of egg	Number of samples		Percent contaminated with:			
			Coliforms		Enterococci	
	Initially	After 1 week at 10°C.	Initially	After 1 week at 10°C.	Initially	After 1 week at 10°C.
A	73	50	10	6	60	*
B	77	50	22	16	69	52
C	74	50	27	26	55	40

* Not analyzed.

and enterococci were isolated from all grades of eggs (Table 3), but the higher incidence of contamination of grade B and C eggs indicates that fecal material was often included in the dirt present on the shells.

Storage for 1 week at 10°C. did not have any appreciable effect on the level of contamination on the shells of grade A and B eggs. These results are in accord with those of other investigators (Haines, 1938; Forsythe *et al.*, 1953) who have concluded that the shell does not support microbial multiplication unless eggs are stored under very humid conditions. The greatest range of contamination (Figure 6) was exhibited by cracked or checked grade C eggs. An assumption that bacterial multiplication was promoted simply by the cracking of the shell was not supported by the results obtained in the following experiments. An area of 1 cm.^{2} on the shell at the equator of naturally clean eggs was inoculated with a paste prepared from deep-litter material. The contaminated shells of some of the eggs were cracked, and, in others, the underlying shell membranes were punctured with a sterile scalpel. The method described earlier was used to determine the numbers of microorganisms on the shell. In addition, the contents were shaken by

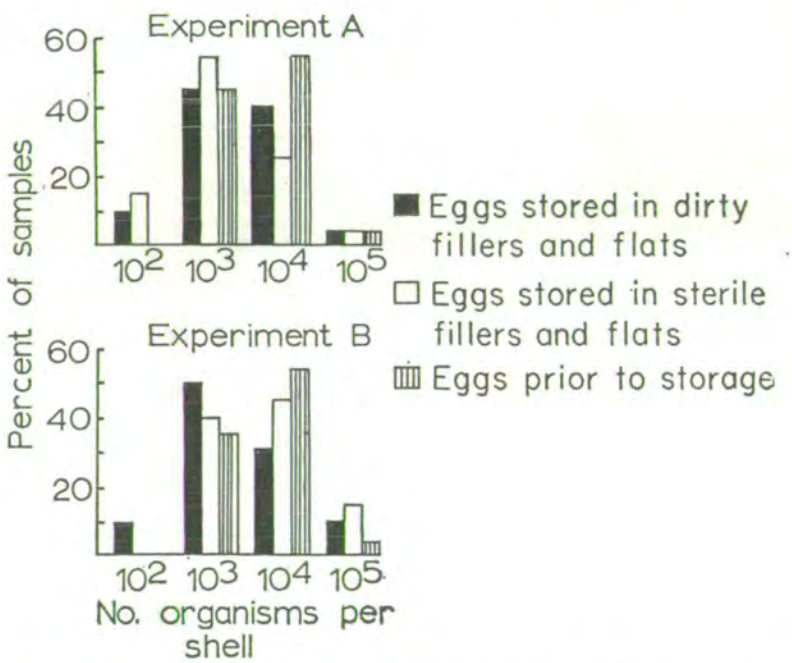

FIG. 4. Frequency distribution of contamination on the shells of eggs (20 per sample) that had been placed on dirty or sterile fillers and flats. Experiment A: eggs stored for 1 week at 10°C. Experiment B: eggs stored for 3 days at 20°C. and "sweated" on 2 occasions.

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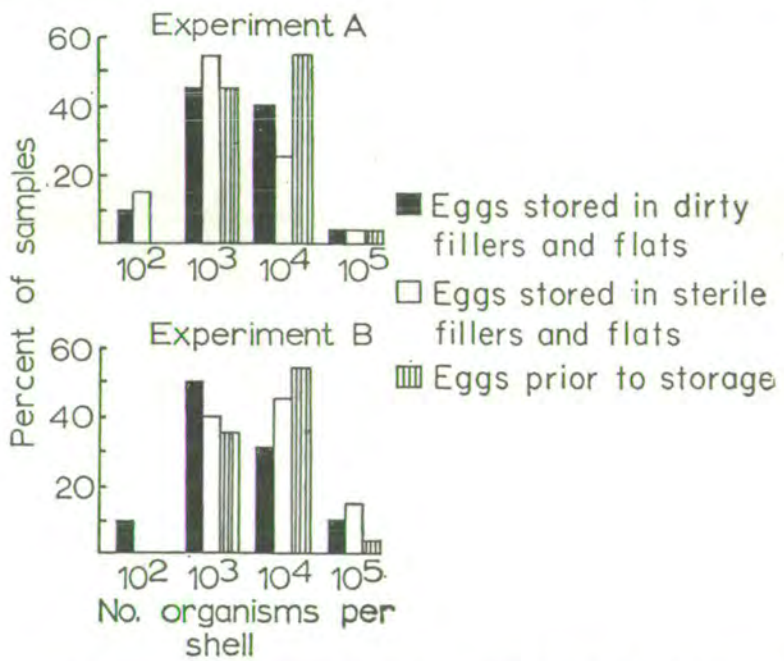

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TABLE 4.—*The incidence of contamination of egg content after various shell treatments*

	Egg shell:		
	Contaminated[1]	Contaminated and shell cracked	Contaminated, shell cracked, and shell membranes punctured
Number of contents examined	24	24	23
Percent contaminated	17	54	74
Range of contamination[2]	10–1,800	50–3,380	50–25,000
Average level of contamination	83	262	4,570

[1] A paste prepared from deep litter material was applied to an area of 1 cm.[2] on the shell surface.
[2] Number of organisms per contents.

hand in a sterile jar until the yolk and white were blended, and 5, 1 and 0.1 ml. amounts were added to 10 ml. TGEA. The puncturing of the shell membranes, the shell or both did not result in a marked increase in the level of contamination of all the shells. The possibility that a particular fraction of the microflora proliferated at the expense of the others present at the beginning of the experiment was discounted by an examination of 40 colonies randomly selected from each of several plates of TGEA. These were streaked on nutrient agar and, after incubation at 30°C. for 24 hours, stained by the method of Gram. This revealed that arthrobacters and, to a lesser extent, micrococci were dominant throughout the experiment. Thus, it would seem that, in addition to the cracking of the shell, the promotion of bacterial multiplication in checked or cracked eggs is dependent upon factors such as specific types of bacteria and age of egg. Table 4 shows that fracturing the shell and shell membranes increased the incidence of contamination of the contents of the eggs. Since it was necessary to examine the shells of these eggs prior to sampling the inner contents, it was not possible to make the latter examination under strictly aseptic conditions. Nevertheless, these results are worthy of note in relation to the possible lowering of the microbiological quality of egg products by the use of cracked or checked eggs.

More than 600 isolates from fillers, flats, and egg shells were examined. The distribution of the various genera recovered from these materials is given in

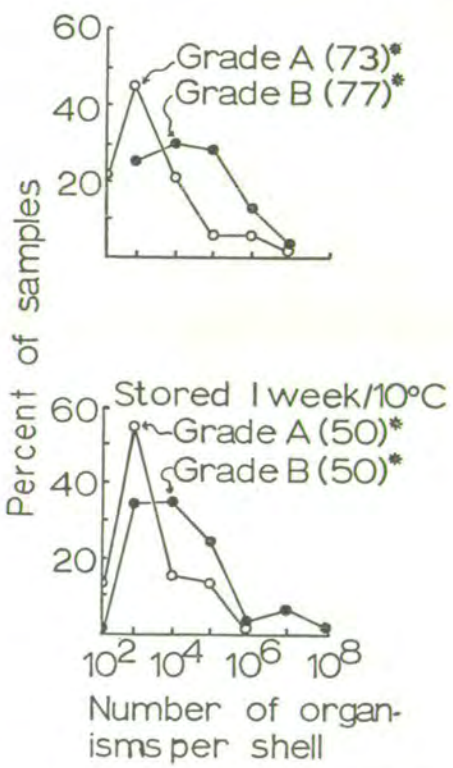

FIG. 5. Frequency distribution of contamination on the shells of eggs.
* Number of eggs examined.

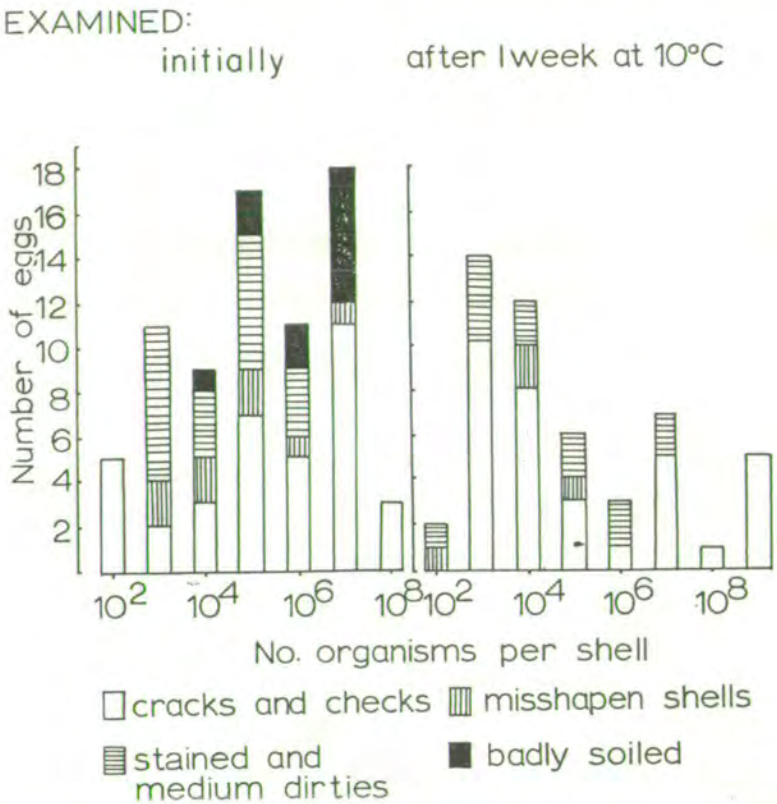

FIG. 6. Frequency distribution of contamination on the shells of various types of grade C eggs.

Table 5. The prevalence of the various types of bacteria growing on TGE agar can be listed as follows: gram-positive cocci > gram-negative rods > Arthrobacter > Bacillus. This sequence differs from that reported by Haines (1938) who found that gram-negative rods (38%), Bacillus (30%), and gram-positive cocci (25%) constituted the major fractions of the 100 isolates which he obtained from the shells of English eggs. Geographical differences and the fact that Haines did not examine fillers and flats may account for the difference between his results and those reported here.

The method of Florian and Trussell (1957) was used to test the rot-producing ability of representative strains of the groups listed in Table 5. A dilute cell suspension was inoculated into the cell of 6 eggs (these were 4 days old at the time of inoculation), and 2 eggs were stored under each of the following conditions: 42 days at 10°C.; 35 days at 10°C. followed by 7 days at room temperature, or 42 days at room temperature. The results are given in Table 6. When the eggs were opened, the rots were identified according to the descriptions given by either Haines (1939) or Florian and Trussell (1957). In addition, the purity of an infection in an egg was checked by subculturing a loopful of the contents on nutrient agar. The eggs inoculated with gram-positive bacteria did not manifest signs of spoilage, nor did their contents yield viable organisms.

TABLE 5.—*The distribution of various types of bacteria on egg shells, fillers and flats*

Organism	Used fillers and flats	Numbers isolated from: Egg grade: A	B	C	Total number isolated	Percent of total
Gram-positive cocci						
Micrococcus	94	49	86	53	282	47.8
Staphylococcus (Mannitol-negative)	2	9	1	14	26	
Gram-positive rods						
Arthrobacter	41	7	22	12	82	12.7
Bacillus	13	—	4	—	17	2.6
Gram-negative rods						
Pseudomonas						
A. Fluorescent	1	2	4	1	8	26.8
B. non-pigmented, proteolytic	10	18	14	13	45	
C. non-pigmented, non-proteolytic	3	9	3	16	31	
Achromobacter	5	2	3	1	11	
Alcaligenes	9	—	3	—	12	
Flavobacterium	1	—	—	—	1	
Cytophaga	1	—	2	—	3	
Unclassified	14	29	9	8	60	
Escherichia	18	6	12	3	39	9.5
Aerobacter	10	8	1	3	22	
Aeromonas	3	1	—	2	6	

TABLE 6.—*Ability of bacteria isolated from egg shells, fillers and flats to produce rots in eggs*

	Number of strains	Changes occurring in eggs inoculated at air cell stored: 10°C. for 42 days: Type of rot	Day detected by candling	10°C. for 35 days followed by room temperature for 7 days: Type of rot	Day detected by candling	Room temperature for 42 days: Type of rot	Day detected by candling
Micrococcus	4	—	—	—	—	—	—
Staphylococcus	3	—	—	—	—	—	—
Arthrobacter	6	—	—	—	—	—	—
Bacillus	2	—	—	—	—	—	—
Escherichia	4	—	—	—	—	—	—
Aerobacter	3	+	—	BR	42	BR	18
Aeromonas	2	BR	42	BR	42	BR	18
Pseudomonas:							
Fluorescent	1	FGR	—	FGR	—	FGR	—
Non-pigmented proteolytic	6	GR	42	GR	42	GR	18
Non-proteolytic	2	+	—	YR	42	YR	18
Achromobacter	2	+	—	GR	42	GR	18
	2	—	—	—	—	—	—

—: no change in appearance of egg and no organisms recovered.
+: only a slight change in appearance of egg and viable organisms recovered.
BR: black rot type 1 of Haines (1939). The following were identified with descriptions given by Florian and Trussell (1957): FGR, fluorescent green rot; GR, green rot; YR, yellow rot.

A similar situation was observed with eggs that had been inoculated with 4 strains of *Escherichia* and 2 out of 4 strains of *Achromobacter*. With the other gram-negative bacteria, viable organisms were recovered from the contents of all of the eggs. Thus, it would seem that the majority of gram-negative bacteria present on the shell and on egg packing materials are potential colonizers of the contents of eggs. The rate of colony formation, as judged by candling, was slowest in eggs held at 10°C. Moreover, the changes produced in these eggs were not as distinct or characteristic for a particular organism as were those in eggs that had been stored at room temperature either briefly or for the duration of the experiment. The rots obtained in the latter group were used for the identification given in Table 6.

The data presented in Table 5 can be used to deduce the most probable sources of contamination to which eggs are exposed. Members of the genus *Micrococcus* are widely distributed in dust and on the surfaces of inanimate objects (Abd-el-Malek and Gibson, 1948; Baird-Parker, 1963). The same authors considered that mannitol-negative strains of *Staphylococcus* are common residents on the skin of warm blooded animals. Thus, the cultures isolated in this study may have originated on the skin of hens or egg-handlers. Rosser (1942) found that eggs handled normally were more heavily contaminated than those handled under aseptic conditions. *Arthrobacter*, which was not isolated by Haines (1938), is among the numerically predominant genera of microorganisms in soil (Topping, 1937). Evidence of contamination with fecal material was also indicated by the isolation of strains of *Escherichia* that produced acid and gas from lactose at 44°C.

Surprisingly few isolates of fluorescent pseudomonads were recovered in view of the common occurrence of these organisms in spoiled eggs. As far as can be ascertained, the present study is the first in which *Aeromonas* has been recovered from eggs in the U.S.A., although these organisms have been isolated from Canadian eggs by Florian and Trussell (1957). These investigators identified their isolates with *Proteus melanovogenes*. Two workers in England, Miles and Halnan (1937), gave this name to the organism which they isolated from imported South African eggs. Subsequent examinations (Miles and Miles, 1951; Eddy, 1960) of several of the original isolates have shown that, because of their polar flagellation and their utilization of glucose under anaerobic conditions, these organisms should be included in the genus *Aeromonas*.

The properties considered for identification of the aerobic, gram-negative bacteria are listed in Table 7. Possible practical implications of the relatively heavy contamination of egg shells with the types of organisms were suggested by the results obtained from an examination of the contents of stored eggs. Fifty-three eggs were stored at 10°C. for 4–7 months after they had been submerged for 5 minutes in cold (10°C.) sterile distilled water. Frequent candling did not reveal evidence of microbial spoilage. Similarly, the contents of the broken-out eggs did not develop off odor or exhibit features other than those associated with the aging of an egg. Nevertheless, the contents of 26 eggs contained 780 to more than 500,000 bacteria. An investigation of 50 isolates showed that the eggs were contaminated with non-proteolytic members of the genera *Pseudomonas*, *Achromobacter*, and *Alcaligenes*.

Several investigators (Haines, 1939; Alford *et al.*, 1950; Florian and Trussell, 1957) have isolated non-proteolytic strains of the coli-aerogenes group of bacteria, pseudomonads, achromobacter, and alcali-

TABLE 7.—*The properties of the obligate aerobic, gram-negative bacteria isolated from egg shells and egg-packing materials*

Genus	Group	Number of isolates	Flagellation	Glucose[1]	Oxidase	Hydrolysis of:			H_2S Production[3]	Pigment[4]
						Arginine[2]	Gelatin	Egg white[3]		
Pseudomonas	A	8	Polar		+	+	1*	1	1	Fluorescent green
Pseudomonas	B	45	Polar	A, a or K	41	1	+	+	+	—
Pseudomonas	C	31	Polar	A, a or K	23	4	—	—	19	—
Achromobacter		11	Peritrichous	A or a	4	—	2	2	4	—
Alcaligenes		12	Peritrichous	K	1	—	3	3	1	—
Flavobacterium		1	Peritrichous	A	—	—	—	—	—	Yellow
Unclassified		60	Non-motile	A, a or K	d	d	d	d	d	—

[1] Tested in the medium of Hugh and Leifson (1953): A = strong acid reaction; a = weak acid reaction; K = alkaline reaction.
[2] Medium of Thornley (1960).
[3] The dissolution of a piece of heat-coagulated egg white in 1% (w/v) casitone water was regarded as a positive result; the blackening of a piece of lead acetate paper suspended over this medium was considered to be evidence of H_2S production.
[4] Tested on the medium of King *et al.* (1954) and milk agar.
* Numerals = numbers of positive strains. + = all strains positive. — = all strains negative. d = different reactions.

genes from eggs which did not exhibit any of the features normally associated with microbial spoilage. In other instances (Turner, 1927; Spanswick, 1932; Levine and Anderson, 1932; Richard and Mohler, 1950), bacterial contamination has been associated with the contents of eggs that differed from sterile eggs only in the possession of an off-odor or off-flavor. Both Brooks (1960) and Board (1962) have found that non-spoilage organisms of this type give a pattern of multiplication in eggs similar to that of spoilage organisms. The incidence of such contamination in commercial eggs together with its influence on the level of contamination of egg products would appear to be worthy of further exploration. It is pertinent to note that such contaminated eggs were recovered occasionally from egg breaking plants by Johns and Berard (1945, 1946).

Only one strain of *Salmonella*, *S. senftenberg*,[5] was isolated in this study. This organism was recovered from the shell of a lightly soiled egg.

SUMMARY

Colony counts obtained on tryptone glucose extract agar were used to assess the level of contamination on the shells of eggs and egg-packing materials entering an egg grading and candling station. The level of contamination ranged from 10^2–10^8 organisms per egg shell. Coliforms and enterococci were isolated from all grades of eggs, but the highest incidence of contamination was found on grade B and C eggs. The results obtained from an examination of new, used, and dirty flats revealed that these become heavily contaminated during use, especially if they are soiled with albumen or yolk. The recovery of coliforms from 31% and 55% respectively of the used and dirty flats indicate that these are often exposed to material of fecal origin. There were no marked differences in the level of contamination of new, used, or dirty fillers; the majority of samples harbored about a million organisms per filler. There was no demonstrable increase in the level of contamination on the shells of naturally clean eggs when these were placed in dirty fillers and flats and stored under various conditions. Of 33 egg cases, all but one gave counts in the range 10^5–10^8 organisms per bottom (interior surface) of case. The unpacking of cases was found to produce heavy aerial contamination. An examination of more than 600 organisms isolated from egg shells, fillers, and flats indicated the following to be the most

[5] The authors are indebted to Miss Alice Moran, National Animal Disease Laboratory of the U.S.D.A., Ames, Iowa, for the identification of this organism.

common contaminants: gram-positive cocci, gram-negative rods, *Arthrobacter*, and *Bacillus*. It was concluded that dust, soil, and fecal material are the usual sources of contamination to which eggs and egg-packing materials are exposed.

ACKNOWLEDGMENTS

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REFERENCES

Abd-el-Malek, Y. and T. Gibson, 1948. Studies in the bacteriology of milk. II. The staphylococci and micrococci of milk. J. Dairy Res. 15: 249–260.

Alford, L. R., N. E. Holmes, W. J. Scott and J. R. Vickery, 1950. Studies in the preservation of shell eggs. 1. Nature of wastage in Australian export eggs. Australian J. Appl. Sci. 1: 208–214.

Baird-Parker, A. C., 1963. A classification of micrococci and staphylococci based on physiological and biochemical tests. J. Gen. Microbiol. 30: 409–427.

Barnes, E. M., 1956. Methods for the isolation of fecal streptococci (Lancefield Group D) from bacon factories. J. Appl. Bact. 19: 193–203.

Board, R. G., 1962 A study of bacterial infection of the hen's egg. Ph.D. thesis. University of Edinburgh, Scotland.

Brooks, J., 1960. Mechanism of the multiplication of *Pseudomonas* in the hen's egg. J. Appl. Bact. 23: 499–509.

Eddy, B. P., 1960. Cephalotrichous, fermentative gram-negative bacteria: the genus *Aeromonas*. J. Appl. Bact. 23: 216–249.

Eddy, B. P., 1962. Further studies on *Aeromonas*. Additional stains and supplementary biochemical tests. J. Appl. Bact. 25: 137–146.

Florian, M. L. E., and P. C. Trussell, 1957. Bacterial spoilage of shell eggs. IV. Identification of spoilage organisms. Food Tech. 11: 56–60.

Forsythe, R. H., J. C. Ayres and J. L. Radlo, 1953. Factors affecting microbiological populations of shell eggs. Food Tech. 7: 49–56.

Haines, R. B., 1938. Observations on the bacterial flora of the hen's egg with a description of new species of *Proteus* and *Pseudomonas* causing rot in eggs. J. Hyg. Camb. 38: 338–355.

Haines, R. B., 1939. Microbiology in the preservation of the hen's egg. Spec. Rep. Food Invest. Bd., Lond., No. 47. London: H.M.S.O.

Hugh, R., and E. Leifson, 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bact. 66: 24–26.

Johns, C. K., and H. L. Berard, 1945. Further bacteriological studies relating to egg drying. Sci. Agric. 24: 551–565.

Johns, C. K., and H. L. Berard, 1946. Effect of certain methods of handling upon the bacterial content of dirty eggs. Sci. Agric. 26: 11–15.

King, E. O., M. K. Ward and D. E. Raney, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44: 301–307.

Kovacs, N., 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature, 178: 703.

Levine, M., and D. Q. Anderson, 1932. Two new species of bacteria causing mustiness in eggs. J. Bact. 23: 337–347.

Miles, A. A., and E. T. Halnan, 1937. A new species of microorganism (*Proteus melanovogenes*) causing black rot in eggs. J. Hyg. Camb. 37: 79–97.

Miles, E. M., and A. A. Miles, 1951. The identity of *Proteus hydrophilus* Bergey *et al.* and *Proteus melanovogenes* Miles and Halnan and their relation to the genus *Aeromonas* Kluyver and van Niel. J. Gen. Microbiol. 5: 298–306.

Rhodes, M. E., 1958. The cytology of *Pseudomonas* spp. as revealed by a silver-plating staining method. J. Gen. Microbiol. 18: 639–648.

Richard, O., and A. Mohler, 1950. Die Microbiologie der "Heueier." Mitt. Lebensm. Hyg. Bem. 41: 168–180.

Rosser, F. T., 1942. Preservation of eggs. II. Surface contamination on egg-shell in relation to spoilage. Canad. J. Res. 20D: 291–296.

Spanswick, M. P., 1932. The cause of mustiness in eggs. Amer. J. Publ. Hlth. 20: 73–74.

Taylor, W. I., 1961. Isolation of salmonellae from food samples. V. Determination of the method of choice for enumeration of *Salmonella*. Appl. Microbiol. 9: 487–490.

Taylor, W. I., and J. H. Silliker, 1958. Isolation of salmonellae from food samples. III. Dulcitol

lactose iron agar, a new differential tube medium for confirmation of microorganisms of the genus *Salmonella*. Appl. Microbiol. 6: 335–338.

Thornley, M. J., 1960. The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bact. 23: 37–52.

Topping, L. E., 1937. The predominant microorganisms in soils. I. Description and classification of the organisms. Cent. F. Bakt. II Abt. 97: 289–304.

Turner, A. W., 1927. Achromobacter perolens (*n.sp.*) The cause of "mustiness" in eggs. Australian J. Exp. Biol. Med. Sci. 4: 57–60.

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